ISOLATION AND CHARACTERIZATION OF A PEANUT PROMOTER

By

DENIS VADIMOVICH BELIAEV

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A peanut (*Arachis hypogaea L.*) promoter was cloned and characterized. To accomplish this, an abundantly expressed pod-specific gene (POD3) was identified and its cDNA cloned using differential display. The POD3 mRNA amplified during a differential display experiment was tested for gene expression analysis in pod and seed tissues. By Northern hybridization, it was found to accumulate in pods but not in seeds or leaves. The complete sequence of POD3 mRNA was determined by 5' RACE experiment and primer extension. It contains a 65 amino acid open reading frame homologous to metallothionein-like genes. A genomic clone containing POD3 coding region and 1180 bp upstream regulatory region was isolated from a custom-made partial genomic library. POD3 has three exons and two introns. The genomic clone sequences upstream of the first, the second and the third exon of POD3 were fused in frame for translation with a β-glucuronidase (GUS) reporter, producing constructs PODGUS1, PG1 and B5 which were expressed in tobacco protoplasts. The construct PODGUS1, containing POD3 sequences

upstream of the first exon, exhibited the highest levels of GUS expression, exceeding those obtained from 35S promoter of cauliflower mosaic virus. B5 was introduced into alfalfa and GUS expression was detected in leaves. This novel promoter may be useful for peanut genetic engineering aimed at reducing aflatoxin contamination by expressing an antifungal compound in pods but not in edible seeds, or it can serve to improve other pod characteristics and help improve understanding of peanut gene regulation.

CHAPTER 1
INTRODUCTION

The overall goal of the project was to isolate and characterize a promoter capable of expressing an antifungal and/or other genes in pods, but not seeds, of transgenic peanut. A gene expressed in pods, but not seeds, was isolated by differential display. The corresponding genomic copy was cloned from a genomic library and used to make and express three promoter constructs in heterologous plant systems. This promoter construct may be of value for genetically engineering peanuts that express antifungal proteins in pods but not in seeds. It seems important to express the foreign anti-fungal protein in the peanut pods and not in the seeds because peanuts are a popular food, especially for children. An antifungal gene expressed in a pod can produce an antifungal barrier protecting the seed, and promote food safety without being expressed in the seed. This will promote consumer acceptance and facilitate approval of this transformed peanut variety for commercial production. This technology is expected to reduce colonization of peanut pods with *Aspergillus flavus*, which produces aflatoxin.

The Aflatoxin Problem

Aflatoxin, a most poisonous natural organic compound, is produced by the fungus, *Aspergillus flavus.* This fungus colonizes peanut pods and aflatoxin contaminates peanuts in the soil so that they cannot be used as food or animal feed. In humans and animals, a microsomal enzyme cytochrome P450 converts aflatoxin B_1 (AFB_1), the most toxic of aflatoxins, into the highly reactive aflatoxin B_1-8,9 epoxide. The latter is

metabolized in several ways: 1) It is oxidized to inert AFB_1-GSH by glutathione-transferase, 2) It is hydrated by epoxide hydrase which appears to play a relatively minor role in toxicity, 3) It binds to guanine residues in DNA, followed by hydrolysis of glycoside bond, and 4) It intercalates between DNA strands. The interactions of AFB_1 and its epoxide with DNA have been shown to cause G→T mutations in liver leading to liver carcinoma. The other diseases thought to be associated with aflatoxin ingestion are kwashiorkor, cirrhosis, and Reye's syndrome (Keenan and Savage, 1994).

Peanut has defense mechanisms against fungal infection. Among them there are mechanical barriers--pod and seed coat. Mechanical damage during digging and insect invasion significantly increase fungal colonization (Keenan and Savage, 1994; Azaizeh et al., 1989). Normally, the growing fruit releases phytoalexins that prevent fungal growth in pod. However, water activity greater than 0.97 is required to keep phytoalexin levels high enough to maintain seeds aflatoxin-free. Fungus also does not grow if water activity is lower than 0.85. The water activity is the ratio of the water amount in the tissue to the water amount in the tissue if it is saturated with water. Thus, there is a "window of susceptibility" within which fungus can attack (Keenan and Savage, 1994) giving a relationship between drought stress and aflatoxin contamination of peanut. According to Cole et al. (1995), the special conditions of 26-30°C and drought are required for *A. flavus* colonizing peanut pods to produce aflatoxin.

Peanut Fruit Development

Peanut has aerial flowers and subterranean fruits, which are its characteristic features. Flowers wither after pollination, and then the embryo undergoes only a few (3-4) divisions before the basal tissue of the ovule starts to divide and form carpophore,

commonly called peg, which rapidly elongates and grows toward the soil bearing the fertilized ovules in its tip. It enters the soil and travels about 5 cm and then pod development begins. The peg tip takes horizontal orientation begins to swell and embryo division resumes (Coolbear, 1994). The stages of the peanut fruit development are summarized in Table 1 (Paik-Ro et al., 2002).

Candidate Genes for Protection Against a Peanut Contaminant *A. flavus*

The antifungal gene can code for a protein inhibiting fungus growth or for an enzyme which degrades aflatoxin. Besides, targeting of the antifungal genes outside the cell by means of a leader peptide may decrease colonization because fungi will encounter the protein before they disrupt any cells. Over 300 leader peptides for outside targeting are currently known (Dr. K. Cline, University of Florida, Gainesville, FL, personal communication). The introduced antifungal protein should reduce the aflatoxin contamination when natural means of protection do not work during drought or at post-harvest conditions, therefore, it should remain active after pod cells die.

A number of antifungal genes have been recently isolated. To efficiently fight *A. flavus*, a pod-specific promoter should be tested in combination with a variety of antifungal genes in order to choose the most effective gene. A few examples of such genes are given below.

De Bolle et al. (1995) describe two genes coding for the small antifungal peptides from *Mirabilis jalapa* (four o'clock plant) which are highly basic and cysteine-rich. It appears that they are expressed as pre-proteins and are cleaved during their export from the cell. So far, their mechanism of action on fungi and some bacteria remains unknown.

Another group of small antifungal peptides consists of KP1, KP4 and KP6 killer

Table 1. Peanut fruit development stages.

Days after flowering	Seed stage	Pericarp	Kernel
10		Peg just penetrates soil surface	
20		Torpedo shape--basal section rapidly enlarges to the maximum length of 1.75 cm	
25		Basal and apical sections enlarge to the maximum length of 3 cm	Small, tightly held by parenchymatous tissue
30	1	Very watery, soft and spongy	Very small, flattened, completely white, mostly seed coat
35	2	Soft, not as watery. Endocarp fleshy--no cracks	Flat, white, just beginning to turn pink on one end
45		Endocarp begins to show cracks	Torpedo shaped, generally pink at embryonic axis end of kernel
55		Endocarp begins to show "cottony" appearance	Torpedo to round shaped, embryonic axis end of kernel pink, other end white to light pink
65	3	Endocarp begins to dry out, cracks more numerous	Round, light pink all over
75		Endocarp white, but begins to show brown splotches	Dark pink at embryonic axis end, light to dark pink elsewhere
85		Many dark brown splotches on endocarp	Large, generally pink all over, seed coat beginning to dry out
95	4	Endocarp almost completely brown	Dark pink, may show imprint of pericarp on seed coat in places, seed coat drying out
105		Black splotches appear on endocarp	Same as above
115		Black splotches over at least one half of the pericarp. Seed coat beginning to turn brown.	
125		Black splotches throughout pericarp. Seed coat almost all brown, imprint of pericarp seen over large part of kernel	

toxins of the *U. maydis* virus (UmV). Park et al. (1996) have obtained transgenic tobacco plants secreting amounts of KP4 toxin sufficient to kill all fungi susceptible to KP4 (Park et al., 1996). KP toxins are acting on cells by blocking specific Ca^{2+} channels. Transgenic carrot, but not cucumber, possessing genes of three different chitinases resulted in increased resistance of plants to three fungi species (Punja and Raharjo, 1996). Chitin and β-1,3 glucan are two major polysaccharides of fungal cell wall, and transformation of plants with both β-1,3 glucanase and chitinase gives better results than insertion of either of these genes alone (Punja and Raharjo, 1996). Chitinases, chitosanases and other antifungal proteins isolated from peanut (Ye and Ng, 2001; Mathivanan et al., 1998; Cuero and Osuji, 1995) are attractive candidates for genetic engineering of fungi-resistant peanuts because they are already present in peanut and, instead of expressing a foreign gene, transformed plants overexpressing that useful gene may be easier to get approved for human consumption.

Osmotins and osmotin-like proteins have been shown to increase disease tolerance in plants. Overexpression of its own osmotin-like protein, pA13, made potato plants more resistant to the late blight fungus *Phytophthora infestans*. However, when antisense pA13 mRNA was expressed, osmotin-like mRNA level was decreased, but it did not change disease resistance (Zhu et al., 1996). There is also growing interest in microbial resistance mediated by small peptides. Four groups of them were identified: the magainins, the cecropins, the defensins and the proline-rich peptides. In addition to native peptides, synthetic peptides are being made and tested for antifungal activity (Powell et al., 2000; Powell et al., 1995). Alpha-amylase inhibitors have recently been isolated from several plants. They often specifically inhibit α-amylases of fungi and insects but not of

animals and humans (Fakhoury and Woloshuk, 2001). These proteins are usually small (under 40 kDa) and the structure of some of them is well-known, which may allow some protein engineering.

A Review of Selected Tissue-specific Control Elements

In eukaryotes, gene expression depends on certain DNA sequences, which are usually recognized by proteins, called transcriptional activators or transcriptional repressors, interacting with the RNA polymerase II initiation complex. These sequences called control elements may be quite short (6 bp or less) and their locations with respect to the transcription start site are diverse and may even be on a different chromosome (Germann et al., 1994). Enhancers and silencers are the two types of control elements. Enhancers interact with transcriptional activators and upregulate the transcription, and and silencers interact with transcriptional repressors and downregulate the transcription, respectively. They determine the rate of transcription of a given gene specific to tissue, developmental stage and environmental stimuli. Some relatively well studied tissue-specific control elements (boxes) are discussed below.

The CArG Box

CArG box has the consensus sequence $C(A/T)_6GG$. In the ABC model of *Arabidopsis* floral development, the identities of the floral parts are determined by the combinatorial expression of five proteins, AP1, AP2, AP3, PI and AG. These proteins can homo- and heterodimerize in solution, but only selected dimers can bind AP3 gene promoter containing CArG box (Riechmann et al., 1996). Two CArG-like elements are found in the promoter of MHC, the myosin heavy chain gene expressed specifically in heart and pulmonary myocardium (Riechmann et al., 1996). However, one of them,

CArG1, has only low negative effect on MHC transcription, whereas CArG2, which is adjacent to the TATA box, increases transcription about 40-fold over the basal level. An inverted CArG, "GArC", seems to be a negative regulator of a smooth muscle actin gene, because the mutated promoter of the gene is three times more active in cultured aortic smooth muscle cells (Swartz and Owens, 1996). Another report of the same group (Hautmann et al., 1996) demonstrates the role of two CArG boxes in the same promoter that lie closer (within 135 bp) to the transcription start site. CArG boxes are necessary for angiotensin II-mediated activation of the promoter. On the other hand, a smooth muscle myosin heavy chain gene regulatory region necessary for the correct expression pattern of the gene spans 4.3 kb upstream and 11.2 kb downstream of transcription start site and includes a CArG box 1.6 kb downstream of the transcription start site in the first intron of the gene.

The ACGT Core Sequence and the G-box

The ACGT core sequence is highly conserved in the array of sequences able to bind hXBP-1, a bZIP type transcription factor (Hautmann et al., 1996). The promoter element vs-1 of a tomato gene, grp1.8, binds a bZIP protein, VSF-1, although this element does not have an ACGT core sequence characteristic of bZIP binding sites. This element determines xylem specificity of the promoter (Torres-Schumann et al., 1996). A bZIP protein ROM2 from tomato binds both ACGT- and ACCT-containing sequences in early seed maturation promoters (Chern et al., 1996) and functions as a repressor of transcription activated by transcription factor PvALF. The consensus sequence GCCAC(G/C)TCA(G/A)YY is protected from DNAse I digestion in tests of ROM2 binding to the promoters (Chern et al., 1996).

Three groups of plant bZIP transcription factors have been distinguished (Mikami et al., 1995) based on their specificity to the sequences flanking ACGT core. The first group containing EmBP-1, HBP-1a, CPRF-1, CPRF-3, TAF-1, OBF-1 and mLIP15 bind the CACGTG-containing sequence (G-box) better than the GACGTC-containing sequence (C-box). Members of another group, CPRF-2, Opaque2 and TGA1b bind the G- and C-boxes equally well, and a member of the other group, TGA1a protein, binds the C-box better than the G-box (Mikami et al., 1995).

In spinach, the Rca gene is expressed specifically in leaves and is light-regulated. The Rca gene encodes a Rubisco activase and its expression is coordinated with the expression of this enzyme (Orozco and Ogren, 1993). Orozco and Ogren (1993) studied the Rca promoter truncated at the nucleotide -294 relative to the Rca transcription start site. This piece of promoter conferred normal pattern of expression to a reporter gene. They found that the G-box present in the promoter is required for expression of -294 Rca promoter-GUS fusion since a deletion of 10 bp around G-box abolished expression. Also, the expression in leaves appears to be tissue-specific because truncation of the promoter past this G-box resulted in the same level of expression of a reporter gene when 35S enhancer was also used to increase the transcription rate. A conserved GT motif (not to be confused with the GT-box) is present in this promoter 21 bp downstream of G-box. However, 2 bp deletions within this element did not change the expression pattern. A 10 bp deletion including GT motif led to expression in roots while expression in leaves remained the same. These data indicate the presence of another yet unknown site of DNA-transcription factor interaction in this promoter (Orozco and Ogren, 1993). Binding sites for transcription factors are often palindromic. An example of a "stretched" binding

site is F-box, which is responsible for reduction of RBCS3A gene expression in tomato fruit. Its structure is ATGAGANNAGNCTNNTCTCAT (Meier et al., 1995). Interestingly, a protein which binds to it does not seem to interact with a GBF, (g-box binding protein), bound to G-box situated only 21 bp downstream (Meier et al., 1995). A basic Helix-Loop-Helix protein, PIF3, binds to G-boxes of several light-responsive promoters. Phytochrome B binds to PIF3-G-box complex of a light-activated gene only in a PfrB (far red) conformation, and the binding is reversible by far red light, suggesting a direct mechanism for light-activated transcription (Martinez-Garcia et al., 2000). In a rice glutenin gene promoter, a mutated AGCT box reduced a reporter gene expression about four fold in transgenic plants, but a mutation in other promoter elements, PROL, AACA, and GCN4 had a stronger effect. Only GCN4, when put upstream of a minimal promoter in multiple copies, has conferred an expression pattern similar to that of wild-type promoter.

The GT Box

The sequence GGGGTGGGG serves as a binding site for transcriptional activators in MMP-9 (mouse metalloprotein-9) promoter in mouse and human cells (Sato et al., 1993). A promoter of a GTP-binding protein expressed in embryonal carcinoma mouse cells, gb110, contains three enhancer elements, two of which are GC (GGCGGG) and GT (GGGGTGGG) boxes (Hamann et al., 1994). Protein binding GC box is a Sp1-like protein, whereas a GT-I-related protein binds to a GT box of the gb110 promoter. Rice phytochrome A gene promoter has three GT-box motifs each specifically recognized by a transactivator protein GT-2 (Ni et al., 1996).

Progress in Peanut Genetic Engineering

Peanut breeding is the traditional way to improve the crop. An alternative to it is genetic engineering. It may make possible the introduction of foreign genes into plants or modify the expression of existing genes. In both cases the ability to express a transgene in the right tissue and at the right time is an advantage. Plant transformation with foreign genes has become important because 1) a new gene will be stably maintained in subsequent generations; 2) a plant will acquire an additional feature which will not interfere with other functions of a plant (for example, the transformation of potato with Bt toxin only added insect resistance to the Colorado Beetle (Gulina et al., 1994)); and 3) gene dosage may be controlled by breeding. Peanut genetic engineering is a poorly developed area of research; however, it seems promising to use it to decrease *Aspergillus flavus* contamination of peanut pods, as well as introduce resistance to other organisms, such as Colorado beetle.

The legumes are possible to transform using *Agrobacterium*-mediated technology. However, these plants are difficult to regenerate from a tissue culture cell, which has delayed the development of the gene transfer techniques. Many legumes have been transformed via *Agrobacterium*. In a number of reports, soybean cotyledons were treated with *Agrobacterium* in different ways, and adventitious shoots were analyzed for expression of the selectable and screenable markers and for the presence of the transgene in the progeny of the transformed plants. The technique proved to be very plant cultivar- and bacterial strain-specific (Chee and Slightom, 1995). McKently et al. (1995) described a single event of the stable peanut transformation over two generations using *Agrobacterium*. They co-cultivated wounded embryo axes with strain A281 of

Agrobacterium, which has been selected as one of the most tumorigenic strains, and germinated them in soil. The seedlings were assayed for GUS activity and 9% were GUS-positive. Seeds of one transgenic plant (T1) were grown and their seeds (T2), in turn were analyzed for GUS expression and their GUS expression pattern corresponded to 3:1 segregation ratio (McKently et al., 1995). This protocol seems promising because it avoids tissue culture and transient expression of the introduced gene is high enough to screen, rather than select for, transformed plants. However, a reproducible peanut transformation protocol had not been developed (Dr. G. Moore, University of Florida, Gainesville, FL, personal communication). To date there have been several reports on *Agrobacterium*-mediated peanut transformation. The co-cultivation of peanut embryos with *Agrobacterium* remains the technique of choice. The variables affecting the transformation efficiency include plant cultivars, bacterial strains, pre-treatment of the explants with media, phytohormones and polarity of epicotyls during co-cultivation (Egnin et al., 1998; Rohini and Sankara Rao, 2001). Wounded tobacco leaf extract was added to co-cultivated *Agrobacterium* and plant explants in order to induce *vir* genes of *Agrobacterium* involved in T-DNA transfer (Cheng et al., 1996).

Other transformation techniques are also being tried; one of them is microprojectile bombardment. Particles (microprojectiles) of gold, tungsten or other materials coated with DNA (linear or plasmid) are bombarded into cultured plant cells or tissue. This method is not species-specific, and the presence of a cell wall is not usually an inhibitory obstacle for efficient transformation. The disadvantages include high cost of the equipment, low frequency of transformation and a low repeatability level. Using microprojectile bombardment, the Ozias-Akins group was able to transform peanut

embryogenic calli with the *Bacillus thuringiensis* cryIA(c) gene (Singsit et al., 1997) and the tomato spotted wilt virus (TSWV) N gene (Yang et al., 1998) and regenerate transgenic plants. The N gene encodes a nucleocapsid protein (Yang et al., 1998). The amount of the anti-insect gene expression inversely correlated with survival rates of the larvae, by they did not report an increase in resistance to TSWV. When antisense TSWV N gene was introduced in peanut by another group, field resistance was observed. However, out of 327 transformed lines, only two were fertile (Magbanua et al., 2000).

Metallothionein-like Genes in Plants

A peanut gene POD3 isolated in our laboratory belongs to the metallothionein-like gene family. Metallothioneins are proteins rich in cysteine and able to bind heavy metals such as Cu^{+}, Zn^{2+} and Cd^{2+}. Metallothioneins are small (<100 amino acids) and their conserved regions are located at the N– and C-termini. The conserved Cys-Xaa-Cys motifs are believed to bind heavy metals. Class I and class II metallothioneins are gene-encoded, whereas class III metallothioneins are poly(-glutamyl-cysteinyl)glycines and their derivatives. They are enzymatically biosynthesized (Robinson et al., 1993).

A number of plant metallothionein-like genes have been isolated. Mutation cup1-1 in a metallothionein gene of *Arabidopsis*, expressed in roots, led to copper sensitivity (van Vliet et al., 1995). Messenger RNA corresponding to JET12 cDNA from *Sambicus nigra*, or common elder, accumulates in the abscission zones of leaves and in senescent leaves. It is induced by ethylene, but the role of the encoded protein remains unclear (Coupe et al., 1995). The E_c protein from wheat germ is the plant metallothionein studied in most detail. It binds Zn^{2+} at the ratio of approximately 5:1. The E_c mRNA accumulates in immature embryos, but only 5% of Zn^{2+} present in mature wheat embryos is bound to

E_c, which contradicts the idea of E_c as Zn^{2+} storage protein. A metallothionein-like gene from barley is down-regulated by ABA, but E_c protein expression is up-regulated by this plant hormone, and promoters of both genes contain an ABA-responsive element (Robinson et al., 1993). Choi et al. (1996) described a metallothionein-like gene expressed in leaves of *Nicotiana glutinosa*. Its expression is induced by wounding and TMV infection, as well as by elevated levels of Cu^{2+} (Coupe et al., 1995). Giordani et al. (2000) reported two metallothionein-like genes from the seagrass *Posidonia oceanica* induced by copper and cadmium. Serial analysis of gene expression (SAGE) of the developing rice seedlings has shown metallothionein as the most highly expressed gene. In this tissue, its mRNA is almost twice as abundant as the globulin mRNA (Matsumura et al., 1999).

The proposed functions of metallothioneins include sequestering and detoxification of excess heavy metals, as well as storage of heavy metals when they are in short supply, free radical scavenging, pathogen defense and leaf senescence. Four types of metallothioneins have been identified in mammals. In yeast, cultured mammalian cells and transgenic mice, overexpression of the metallothioneins led to protection from free radicals, and gene knockouts led to free radical sensitivity (Ghoshal and Jacob, 2001).

The Specific Objectives of the Dissertation

Objective 1. To isolate a gene differentially expressed in pods and not in seeds.

Objective 2. To isolate the POD3 genomic clone and analyze its structure.

Objective 3. To express reporter gene constructs under control of POD3 sequences in order to map the promoter regulatory sequences and to select a promoter construct for peanut genetic engineering.

CHAPTER 2
MATERIALS AND METHODS

Differential Display

Differential Display (Liang and Pardee, 1992) was conducted to identify genes expressed in peanut pods but not in seeds. Total RNA was isolated from the field-grown peanut pods and seeds (maturity ranging from Seed Stage 2 to 3, see: Table 1) of cultivar Altica using the phenol/SDS method according to Ausubel et al., (1994). The differential display experiment was carried out at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR), Differential Display Core Laboratory. DNAse-treated RNA was reverse transcribed with each of the four reverse primers followed by polymerase chain reaction (PCR) amplification with the same reverse primer and one of the five forward 10 nt long primers (see Table 2) in the presence of α^{35}S-dCTP. The resulting 20 pairs of the pod and seed RNA amplification products were run in adjacent lanes of the denaturing 8% polyacrylamide gel, the gel was fixed on Whatman paper and X-ray film was exposed on the gel for 3 days. The dried gel and autoradiograph were then provided to me for subsequent re-amplification and cloning of differential display products.

Table 2. Primers used for differential display. All primers are listed 5'-3'. (AGC) denotes any of A, G, or C nucleotides randomly incorporated.

FORWARD PRIMER	*SEQUENCE*	*REVERSE PRIMER*	*SEQUENCE*
1	GTTGCGATCC	T_{12}(AGC)G	TTT TTT TTT TTT(AGC)G
2	CAAACGTCGG	T_{12}(AGC)A	TTT TTT TTT TTT(AGC)A
3	AGGTGACCGT	T_{12}(AGC)T	TTT TTT TTT TTT(AGC)T
4	GACCGCTTGT	T_{12}(AGC)C	TTT TTT TTT TTT(AGC)C
5	AGCCAGCGAA		

Extraction of the Differential Display Products from the Gel

The autoradiograph of the differential display gel was aligned with the gel to identify areas of the gel containing a band of interest which was cut out of the gel with a scalpel, together with paper bound to the gel. The excised pieces were boiled for 15 min in 100 µl of deionized water in 1.5 ml microcentrifuge tubes; the tubes were then centrifuged at 15,000g and the liquid was transferred to other tubes. One tenth volume of 3M sodium acetate, 25 mg of DNAse-free glycogen (Boehringer Mannheim, Indianapolis, IN) and 0.45 ml of 100% ethanol were added and the DNA was precipitated overnight at -20^{0}C. The tubes were centrifuged at 15,000g for 15 min at 4^{0}C, the pellet was washed with 85% ethanol, air-dried and resuspended in 10 µl of water.

Polymerase Chain Reaction

For amplification of the differential display bands, DNA subcloning and transgene detection in transformed alfalfa, PCR was performed according to Sambrook et al., (1989). Four microliters of the DNA extracted from differential display bands were re-amplified for subsequent T-A cloning with the same primers as those used for the differential display. Forty cycles of PCR consisted of the following steps: denaturation at 92^{0}C for 30 sec, annealing at 37^{0}C for 1 min, synthesis at 70^{0}C for 2 min. For PCR-mediated subcloning, 10 ng to 1 µg of plasmid DNA was amplified for 25-35 cycles with primers described in "Cloning of POD3-GUS Constructs" section of Results and Discussion Chapter. The cycle parameters were the same as above except the annealing temperatures were determined experimentally to get maximum synthesis of the correct DNA fragment. To confirm the presence of neomycin phosphotransferase II (NPTII) in regenerated alfalfa plants, 1 µg of alfalfa genomic DNA was amplified for 35 cycles with

the cycle parameters described above with a change in annealing temperature to 57^0C with primers 5' NPT-A (ATT GCA CGC AGG TTC TCC) and 3' NPT-B (GAT GCG CTG CGA ATC GGG).

Northern Hybridization

For northern hybridizations, total RNA was isolated from peanut tissues by the phenol/SDS Method (Ausubel et al., 1994). RNA electrophoresis was conducted according to Sambrook et al., (1989) in gels containing 2% agarose and 7.4% formaldehyde followed by RNA transfer onto Magnacharge nylon membrane (Micron Separations Inc., Westborough, MA). All hybridizations were carried out in a rotary incubator (Robbins Scientific®, Sunnyvale, CA) in cylindrical tubes. Nylon membranes were pre-hybridized in a buffer consisting of 7% SDS, 0.25 M sodium phosphate, pH 7.4, 1 mM EDTA, 10 g/l bovine serum albumin (BSA) and 50 mg/l low molecular weight salmon sperm DNA, for 3 hours or more at 60^0C, then a denatured ^{32}P-labeled probe was added and hybridization was carried out overnight at the same conditions as pre-hybridization. For probe labeling, DNA fragments were cut out of the vector with restriction enzymes, separated from the vector on an agarose gel and purified from the gel by Qiaex gel extraction kit, (Qiagen Inc., Valencia, CA). Probes were then ^{32}P-labeled by using a Tag-It kit, (Bios, Inc.) The hybridized filters were washed thrice in 0.1X SSC buffer (20XSSC is 3M sodium chloride, 0.3M sodium citrate pH 7.0) with 0.1% SDS for 20 min at the hybridization temperature. The membranes were exposed to an X-ray film for 1-3 days and/or analyzed by a PhosphoImager, Molecular Dynamics, Sunnyvale, CA. Adobe Photoshop 5.5 was used to quantify the intensity of hybridization signals.

Genomic DNA Isolation.

For Southern hybridizations and a genomic library, a scaled-up modification of a procedure described in (Dellaporta et al., 1983) was used to isolate peanut genomic DNA from 20 g of fresh young leaves. The concentration of DNA was determined spectrophotometrically by measuring OD_{260}. To screen regenerated alfalfa plants for introduced NPTII gene by PCR, alfalfa genomic DNA was isolated from leaves according to a protocol in which a leaf disc was pinched off by the lid of a microcentrifuge tube and ground in the tube containing 0.2 ml of the Micro Prep Buffer (0.146 M sorbitol, 0.125 M Tris-Cl pH 7.5, 0.8 M sodium chloride, 0.8% CTAB, 20 mM EDTA, 0.8% N-lauroylsarcosine (sarcosyl) and 10 mM sodium bisulfite) with plastic disposable pellet pestles (Kontes Glass, Vineland, NJ). Then, 0.5 ml of the additional Micro Prep Buffer was added and sample was incubated for 45 min at 65^0C, chloroform-extracted, precipitated with 0.7 volume of isopropanol, washed with 70% ethanol and dissolved in 50 µl of water (Tieman et al., 2001).

Southern Hybridization

Genomic DNA was digested with restriction enzymes in a buffer supplied by the manufacturer at a rate of 3-10 units of enzyme per 1 µg of DNA, incubated for 1 hour; then the same amount of enzyme was added again for an overnight incubation, then loaded on the gel. Reverse transcription-PCR products were directly loaded on the gel. DNA was electrophoresed in agarose gel and capillary transferred (Southern, 1975) to Magnacharge nylon membrane in 20X SSC buffer; the membrane was then dried and UV-crosslinked (Sambrook et al., 1989). Hybridization buffer, radioactive probe

preparation, hybridization and washing were the same as those used for RNA hybridizations.

Plasmid Construction.

A promoterless GUS construct NOPGUS was cloned by inserting *HindIII-EcoRI* fragment of pBI101.3 (Clontech, Palo Alto, CA), containing the promoterless GUS cassette, in pUC19 cloning vector.

To clone PODGUS1, F5 genomic clone was amplified with a forward sequencing primer in the vector and a reverse primer POD3-5d, which had a *BamHI* recognition sequence added to its 5' end. PCR products were resolved on agarose gels, cut out of the gel and DNA isolated from the gel with the Qiaex kit. PCR fragments were cloned into pGEM-T vector (Promega, Madison, WI) according to the manufacturer's protocols.

To clone B5, F5 genomic clone fragments were amplified with a forward sequencing primer in the vector and a reverse primer RES70, which had a *BamHI* recognition sequence added to its 5' end. PCR products were resolved on agarose gels, cut out of the gel and DNA isolated from the gel with Qiaex kit. PCR fragments were cloned into pGEM-T vector. To clone PG1, a *BamHI* site was created in F5 by site-directed mutagenesis using WH908 and WH 909 primers and a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Table 3 contains the primer sequences and locations.

To prepare a vector to clone all these promoters, CaMV 35S promoter was removed from pBI221 (Clontech) by digesting pBI221 with *HindIII-XbaI*, blunting the DNA ends and self-ligation. Then, these promoters were digested with *BamHI* and cloned in *BamHI* site of this vector. All promoter-GUS junctions were verified by sequencing

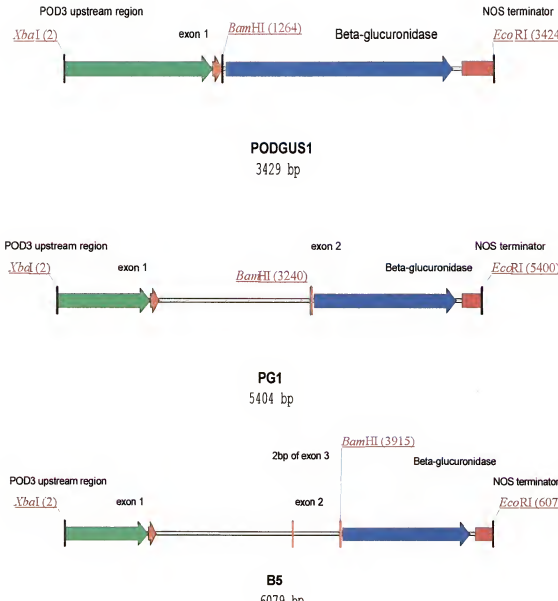


Figure 1. POD3-GUS fusion constructs.

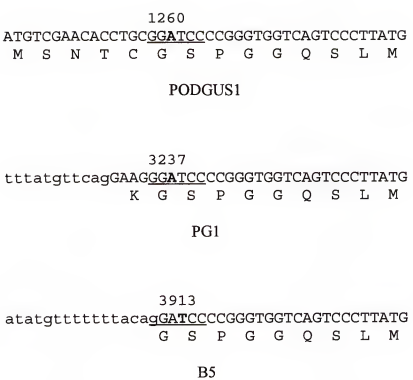


Figure 2. The POD3-GUS junction sequences of the reporter constructs used. Protein-coding sequences are in uppercase letters and intron sequences are in lowercase letters.The translations are below the nucleotide sequences. *BamHI* cloning sites used for cloning POD3 promoters are underlined. The 3' ends of POD3 sequence are bolded and their nucleotide positions (Fig. 8) are typed above the sequences. The ATG triplets at the 3' end of the sequences code for the first methionine (M) in GUS.

(Fig. 2). To insert B5 into pBI121 (Clontech) for *Agrobacterium*-mediated transformation, pBI221 was cut with *HindIII-EcoRI* and ligated with *HindIII-EcoRI* fragment of B5.

Plasmid and Phagemid DNA Isolation, Oligonucleotide Design, DNA Sequencing and DNA Sequence Analysis

Small-scale plasmid DNA isolations were done either by Promega WizardPlus SV miniprep kits or by a polyethylene glycol (PEG) precipitation protocol modified from Sambrook et al., (1989). In the PEG protocol, 3 ml of overnight bacterial culture was spun in a microcentrifuge tubes and the pellets resuspended in 0.2 ml of GTE Buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA); then 0.3 ml of the lysis solution (0.2 M NaOH, 1% SDS) was added and mixed for 5 min by inverting the tube. Next, 0.3 ml of 3 M sodium acetate, pH 4.8 was added, tubes shaken, incubated on ice for 10 min and centrifuged for 10 min. The supernatant was incubated at 37^{0}C for 20 min with 20 mg/l DNAse-free RNAse A (Sambrook et al., 1989), extracted twice with 0.4 ml of chloroform and precipitated with an equal volume of isopropanol at room temperature. DNA was spun down, washed with 70% ethanol, dried and dissolved in 32 µl of water. Then 8 µl of 4 M sodium chloride and 40 µl ice-cold 13% PEG8000 was added and the tubes were incubated on ice for 30 min. The tubes were then centrifuged for 30 min at 4^{0}C, the pellet washed with 70% ethanol and resuspended in 20 µl of water. The single-stranded phagemid DNA of the genomic clone F5 was isolated according to instructions accompanying the Undigested Lambda ZAP II Cloning Kit (Stratagene). Primers for sequencing and PCR were selected by Oligo Primer Analysis Software (Plymouth, MN) except when the primer position had to be in an exact location, then primers were simply made complementary to the binding site. When needed, the restriction sites were added to

5'-ends of the PCR primers. The GCG software package, (Genetics Computer Group, Madison, WI), Vector NTI software (Informax, Inc.), and the NCBI website (www.ncbi.nlm.nih.gov) were used to arrange and analyze sequences. Sequencing was done by either using ABI Prizm BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) or by using fmol DNA Cycle Sequencing System (Promega) according to the manufacturers' instructions. To sequence DNA, a "primer walk" procedure was used, that is, new sequence data were used to design the next primer downstream of a sequenced region, and so on. The primers used for F5 sequencing are listed in Table 4.

Some sequencing reactions were carried out at the Interdisciplinary Center for Biotechnology Research (ICBR) at University of Florida. Gel analysis of the BigDye reactions was done either at the ICBR or at the DNA Sequencing Laboratory of the Center for Mammalian Genetics (CMG) at the University of Florida. For sequencing by fmol DNA Cycle Sequencing System, the samples were analyzed on 8% acrylamide denaturing gels cast and run according to (Sambrook et al., 1989) with one glass plate treated with Gel Slick (FMC BioProducts, Rockland, ME) so that after the plates are separated, the gel will stay on the untreated plate. The gel was then transferred to Whatman paper, vacuum dried and autoradiographed.

Peanut Partial Genomic Library Construction

Thirty micrograms of peanut genomic DNA were digested overnight with *XbaI* and resolved on 0.6% Metaphor (FMC BioProducts, Rockland, ME) agarose gel. DNA fragments of 6-9 kb were cut out of the gel, phenol extracted and precipitated with two volumes of ethanol in the presence of 0.1 M sodium chloride. Of the 1.8 μg of DNA

Table 3. Primers used for subcloning, transcription start site mapping and hybridization.

Name	Sequence	5'position	direction
RTPOD3F	AGCAGCATACATAGCATT	1188	forward
PODHYB	ACCTGCGGAAACTGCGACTGCG	1252	forward
POD3-4d	CGCAGTTTCCGCAGGTG	1267	reverse
POD3-5d	AGGATCCGCAGGTGTTCG	1264	reverse
WH908	GTTCAGGAAGGGATCCAAATACGGT	3225	forward
WH909	CAACACCGTATTTGGATCCCTTCCTGAAC	3253	reverse
RTPOD3R	GTTTCCACGATGTCAA	3266	reverse
RES70	AATGGATCCTGTAAAAAAACAT	3913	reverse
POD3-1d	ACTTCCCATCGTTCTCA	3963	

Table 4. F5 sequencing primers.

Name	Sequence	5' position	direction
DBR8	CGCGCTCGCTACTCAAAC	376	reverse
RES69	GTAGCAGACCCGAAAAA	463	forward
DBR5	GGTGAAAAATCGGAAAG	1936	reverse
RES71	GAACACCTGCGGAAACTG	1248	forward
DBR3	TGCAAATAGAAGTGAAGG	1316	reverse
DBR2	TCATGGAGGACGGTGAAT	2089	reverse
DBF1	TTTGTGACCGATGTGAAC	2536	forward
RES67	AGGTTCTTTGCGTCGAT	2978	reverse
DBF2	TTTGTGCTATATGCCTAA	3152	forward
RES64	TTTATCTTACCGTCAATTTCA	3496	reverse
DBF3	AATAATTACACGGGAAAG	3858	forward
RES53	TGCGGTGCTAACTGCTC	3969	forward
RES58	AGTGCATTTTACTCACACAG	4120	reverse
DBR6	ACGGTTGTTGGTGTTGGA	4318	reverse

recovered from this gel, 1.0 μg was ligated into a *XbaI*-digested and calf intestinal alkaline phosphatase treated Lambda ZAP II cDNA cloning vector . The ligation products were packaged into Gigapack III packaging extract. The library was titered, amplified and screened with a POD3 probe. Inserts of positive clones were excised *in vivo* into Bluescript using XL1-Blue MRF' and SOLR *E.coli* cells and ExAssist helper phage. Lambda ZAP II vector, Gigapack III, bacterial strains and helper phages were all purchased from Stratagene and used according to their protocols.

Poly(A) RNA Isolation

Poly(A) RNA was used for primer extension and 5' RACE experiments to locate the transcription start site. Crude RNA was isolated from 7 g peanut maturity stage 2 pods, (see Table 1) according to phenol/SDS method (Ausubel et al., 1994); however, poly(A) RNA isolation was carried out *in lieu of* LiCl RNA precipitation. To isolate the poly(A) fraction, Dynabeads Oligo $(dT)_{12}$ (Dynal, Oslo, Norway) were used four times, according to the supplied protocol, yielding 2 μg of poly(A) RNA, as was determined spectrophotometrically.

Primer Extension

The protocol developed by Dr. Pring (Yan and Pring, 1997) was modified as described below. Ten picomoles of a DNA primer in 5 μl of water was incubated at 90^0C for 5 min and quickly brought to ice, centrifuged and brought back to the original volume. The primers used were RES58, POD3-1d and POD3-4d. One microliter of 10x T4 polynucleotide kinase buffer, 3 μl of ^{32}P-labeled γ-ATP (3000Ci/mmol) and 1 μl of T4 polynucleotide kinase (Promega) were added and the reaction was incubated for 30 min at 37^0C. Three microliters of the phosphorylated primer was mixed with 10 μl (1 μg) of

poly(A) RNA, 1 µl of 5M NaCl and 50 µl of ethanol, incubated for one hour at -70^{0}C, pelleted, washed and air-dried. The pellet was resuspended in 4.1 µl of water, 1.4 µl of 5X first strand buffer was added followed by denaturation for 5 min at 75^{0}C and annealing for 5 min at 42^{0}C. While the reaction tube was kept at 42^{0}C, 1 µl of 10 mM dNTPs and 0.5 µl of SuperScript II (Life Technologies, Rockville, MD) was added and the reaction was incubated for 15 min. The reaction temperatures varied between 42^{0}C--50^{0}C. The reaction was stopped by addition of 4 µl formamide dye.

Five-prime RACE

A Marathon™ cDNA amplification kit (Clontech) was used to clone the 5' end of POD3 cDNA. The protocol supplied with the kit was followed. One microgram of poly(A) RNA isolated from stage 2 pods was used as template for the first strand of the cDNA synthesis. The gene-specific primers used were RES58 and POD3-1d. In each experiment, the same reverse primer was used for both the first strand synthesis and for PCR. Superscript II (life Technologies, Gaithersburg, MD) was substituted for MMLV reverse transcriptase.

Agrobacterium-mediated Alfalfa Transformation

A protocol (Austin et al., 1994) from Dr. D. Samac of USDA, MN was used. Dark-green leaves of alfalfa cv. Salomac were surface-sterilized for 10 sec in 70% ethanol and for 1-2 min in 1.2% HClO, and 0.05% Tween 20, rinsed three times in sterile water and cut into leaf discs. The disks were inoculated for 20 min with an overnight culture of *Agrobacterium tumefaciens* strain LBA4404 transformed with B5 construct inserted in a T-DNA vector as described above. *Agrobacterium* was transformed with T-DNA vector by Dr. R. Shatters. Bacteria were diluted 1:5 in SHO medium. The SHO medium and other

media used are defined in Table 5 below. Leaf discs were then blotted on sterile Whatman paper and incubated at 24^{0}C for 4 days on B5h plates. After that, the discs were rinsed three times in sterile water and placed on B5h plates again for two days. Then the leaf discs were rinsed three times with sterile water and transferred to B5hKTc selection plates for 2-3 weeks and subsequently to B5hOKTc regeneration plates. After 3 weeks the dark-green embryos were extracted from callus and put on MMSTc plates. The plants which regenerated from these embryos were moved to MMSTc in magenta boxes and subsequently, after establishing roots, to soil pots.

Table 5. Media used for *Agrobacterium*-mediated alfalfa transformation.

SHO medium	Schenk and Hildebrant salts, Schenk and Hildebrant vitamins, 30 g/l sucrose, 0. g/l MES, pH 5.7 with KOH
B5h plates	3.1 g/l Gamborg's B5 salts, 1.0 ml/l 1000X Gamborg's vitamins, 0.5 g/l KNO_3, 0.25g/l $MgSO_4$ x $7H_2O$, 0.5 g/l proline, 30 g/l sucrose, pH to 5.7 with KOH, 8 g/l phytagar, 30 ml/l stock amino-acids and hormones added before pouring
B5h stock amino-acids	For 250 ml: 6.65 g L-glutamine, 0.83 g serine, 0.004 g adenine, 0.083 g L-glutathione, filter sterilized and stored at 4^{0}C
B5h hormones	1 mg/l 2,4-D, 0.1 mg/l kinetin, filter sterilized and stored at -20^{0}C
B5hKTc selection plates	B5h plates with 25 mg/l kanamycin and 500mg/l timentin, carbenicillin or ticarcillin
B5hOKTc	same as B5hKTc but lacking hormones
MMSTc plates	4.3 g/l Murashige and Skoog salts, 1 ml/l 1000X Nitsch and Nitsch vitamin stock, 0.1 g/l myo-inositol, 30 g/l sucrose, pH to 5.7 with KOH, 7.6 g/l phytagar. 500mg/l timentin, carbenicillin or ticarcillin added before pouring

NPTII Enzyme Assay

The neomycin phosphotransferase II (NPTII) assay was conducted on alfalfa plants transformed with the B5 promoter construct to verify plant transformation and it also indicated that the T-DNA had integrated in a transcriptionally active chromosome region. Fresh young leaves were used because the nopaline synthase (*nos*) promoter driving NPTII is constituvely active in this tissue (Bevan et al., 1983). The protein extraction and NPTII assay were done according to Draper et al. (1988). Briefly, the assay consists of a native protein gel electrophoresis followed by incubation of the gel in NPTII reaction buffer with kanamycin and ^{32}P-labelled ATP as substrates. NPTII phosphorylates kanamycin with ^{32}P-labelled phosphate group transferred from ^{32}P-γATP. The gel is then blotted on a special P81 Whatman paper able to selectively bind phosphorylated kanamycin. The ^{32}P-labelled P81 Whatman paper was exposed to both X-ray film and PhosphoImager screen for 1-3 days.

Beta-glucuronidase Activity Measurements

Intact tissue samples were stained by incubation in the GUS staining solution prepared by mixing 1.056 ml of GUS stock solution with 0.144 ml of 25 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) solution in N,N-dimethylformamide and 3 ml of water. The GUS stock solution was prepared by mixing 1 ml of 50 mM potassium ferrocyanide, 1 ml of 50 mM potassium ferricyanide, 1 ml of 10% Triton X-100, 20 µl of 0.5M EDTA, 5 ml of 0.2 M sodium phosphate and 1.98 ml water. The samples were incubated overnight at 37^{0}C and rinsed in 100% ethanol several times until no chlorophyll remained in the samples. The X-Gluc breakdown product is

blue and precipitates inside of cells. The samples were then photographed on color film and the color prints were made.

To quantify GUS activity in alfalfa leaves and tobacco protoplasts, protein extracts were assayed using 4-methylumbelliferyl-β-D-glucuronide (MUG). The GUS enzyme breaks MUG down to 4-methylumbelliferone (4-MU), which is detected by its fluorescence. Alfalfa leaves were homogenized with carborundum in 1.5 ml tubes by plastic disposable pellet pestles (Kontes Glass, Vineland, NJ) in GUS Extraction Buffer (Draper et al., 1988). One milliliter of GUS Extraction Buffer was used per 1 g of tissue. Freshly transformed tobacco protoplasts were centrifuged for 7 min at 4^0C and sonicated in 100 µl of the GUS/LUC Extraction Buffer (Hattori et al., 1992) by 10 pulses 0.5 sec long using an ultrasonic generator. Two hundred ml of the GUS/LUC Extraction Buffer were prepared by mixing 40 ml of 1 M sodium phosphate, pH 7.8, 61 mg of dithiothreitol (DTT), 1.6 ml of 0.5 M EDTA, 10 ml glycerol and 148 ml water. Both alfalfa and tobacco samples were then centrifuged for 5 min at 4^0C. Thirty µl of supernatant was mixed with 45 µl of 2 mM 4-methylumbelliferyl-b-D-glucuronide (MUG) solution in GUS/LUC extraction buffer. Aliquots of 25 µl each were taken before and after one hour incubation at 37^0C. The aliquots were put in 5 ml of 0.2 M Na_2CO_3 and the 4-MU concentration was determined by measuring the solution fluorescence at excitation and emission wavelengths of 365 nm and 445 nm, respectively, on a spectrofluorophotometer (RF500U, Shimadzu Corp., Japan).

Reverse Transcription-PCR (RT-PCR)

To analyze transgenic alfalfa plants for expression of POD3-GUS chimeric mRNA, RT-PCR was conducted on young leaves of transgenic alfalfa. Total RNA was isolated by a procedure from Pawlowski et al., (1994), modified by Il-Ho Kang, Agronomy Department, University of Florida, as follows. In this procedure, 0.2-0.4 g of tissue was ground with mortar and pestle in liquid nitrogen and transferred to a cold microcentrifuge tube by a chilled spatula. It was then mixed with 0.8 ml of hot (90^0C) acidic phenol/extraction buffer (pH 4.5) (1:1 mixture), shaken for 5 min at room temperature and extracted twice with 0.4 ml of chloroform for 30 minutes followed by centrifugation at 12,000g for 15 min at room temperature. The aqueous phase was precipitated with 1/3 volume of 8 M LiCl at 4^0C overnight, centrifuged at 12,000 g for 30 minutes at 4^0C, washed with 2 M LiCl, centrifuged at 12,000g for 5 minutes at 4^0C, washed twice with 70% ethanol and dissolved in DEPC-treated water. The extraction buffer contained 0.1 M LiCl, 0.1M Tris-Cl pH 7.5, 1% SDS and 10 mM EDTA. RNA quality was checked by running it in a 2 % agarose gel with 6% formaldehyde prepared as described in Ausubel et al., (1994). One microgram of total RNA was used as a template for RT-PCR carried out by using Tth polymerase (Promega) according to the manufacturer's protocol. The thermostable Tth polymerase has both reverse transcriptase and DNA polymerase activities so two reactions can be conducted in the same test tube. The first-strand cDNA was amplified by 30 PCR cycles in the presence of 4 mM $MgCl_2$. The cycle parameters were as follows. The denaturation was at 92^0C for 30 sec, annealing was at 56^0C for 30 sec, and synthesis was at 70^0C for 1 min. The reaction products were resolved on 3%

agarose gels and transferred to nylon membranes for Southern hybridization as described above.

Transient Reporter Gene Expression in Tobacco Protoplasts

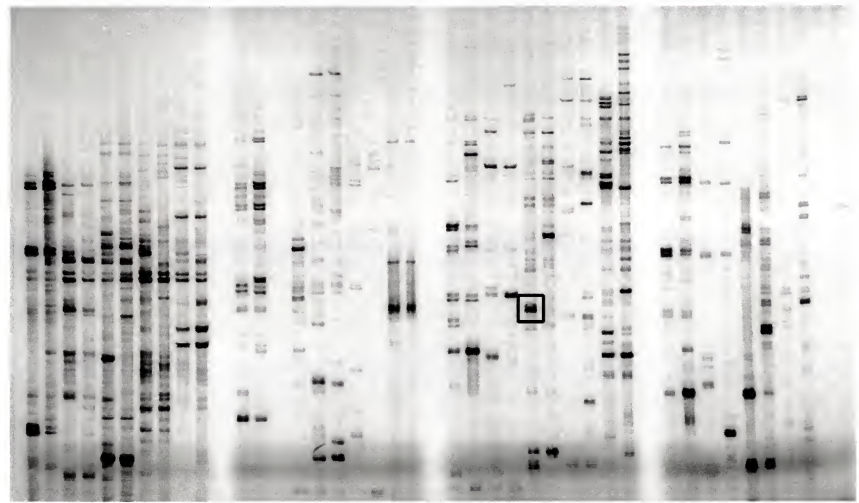
Tobacco protoplasts cv. Samson were generously provided by Dr. E. Czarnecka, Department of Microbiology and Cell Science, University of Florida. Five μg of DNA of the promoter-GUS fusion constructs was mixed with 2.5 μg of pAHC18, a ubiquitin promoter-luciferase construct (Christensen and Quail, 1996). The promoter-GUS constructs used were NOPGUS, PODGUS1, PG1, B5 and pBI221 (Clontech). Polyethylene glycol (PEG)-mediated protoplast transformation followed by incubation for expression of the transiently introduced genes was carried out according to Czarnecka-Verner et al. (2000).

CHAPTER 3
RESULTS AND DISCUSSION

Identification, cloning and characterization of pod-specific cDNAs.

The differential display was chosen as a technique to find a pod-expressed gene because it is relatively fast and does not require cDNA libraries as do differential hybridization techniques (Kiyosue et al., 1994). Differential display was conducted on total RNA from pods and seeds of peanut to identify genes differentially expressed in those tissues. An autoradiogram of the denaturing polyacrylamide gel of the differential display products is shown in Fig. 3. The quality of the differential display reactions can be judged by the presence of the same intensity of most bands in both pod and seed cDNA lanes. These represent RNAs expressed at the same level in both tissue types. These bands indicate that the conditions for differential display reactions with a particular primer pair were the same with both samples being compared and the bands of different intensity in a pair are likely to represent differences in message expression. Bands of the same intensity in both pod and seed lanes may represent housekeeping genes, such as genes for cytoskeleton proteins, histones and ribosomal proteins. On the other hand, if all bands in one lane are weaker than in another lane that was amplified with the same primers but different template RNA, then for some reason one of the reactions did not work well and the bands amplified in another lane are not likely to come from differentially expressed genes. In Fig. 3, lanes amplified by reverse primer T_{12}(AGC)A and forward primer #2 and by reverse primer T_{12}(AGC)C and forward primer #5 were considered not useful in

Figure 3. Differential display gel. The top line lists the four anchored polyT reverse primers and the second line the five forward 10 nt long primers (see Table 2). These primers give 20 primer combinations. Each primer combination was used to amplify both pod and seed mRNAs. These products were run in pairs on the gel with pod on the left and seed on the right. POD3 (boxed) was amplified with T_{12}(AGC)T and 10-mer #3 and was selected for further study.


T12(AGC)G
1 2 3 4 5
T12(AGC)A
1 2 3 4 5
T12(AGC)T
1 2 3 4 5
T12(AGC)C
1 2 3 4 5

identifying differentially expressed cDNAs because of absence of the same intensity bands. Only differentially amplified bands from the lanes containing same intensity bands from two tissues were considered to represent potentially differentially expressed genes. Out of total 578 bands observed on the gel, 30 bands were more intense in pods than seeds and 45 bands were more intense in seeds than pods. Of the 15,000 genes estimated to be expressed in eukaryotic cells (Graf et al., 1997), these 578 cDNAs are only a small subset of possible cDNAs. The possible reasons for such a low fraction are low abundance of many cDNAs, large distances between primers, mRNA secondary structures, to name a few. The specific forward primer sequences also decrease the number of amplified bands. The number of forward primers (5) was limited by number of the gel lanes (40).

Following autoradiography, the gel was aligned with its autoradiographic image on the film and 30 bands that were more intense in pods than seeds as well as 45 bands that were more intense in seeds than pods were cut from the gel. Seed-specific bands were stored for future research on seed-specific genes. DNA was extracted from the bands containing cDNAs preferentially amplified by pod RNA and re-amplified with the primers used for the differential display PCR. The re-amplification products were cloned into pGEM-T vector (Promega). One clone derived from each band was used as a probe for northern hybridizations with RNA from different tissues of peanut to verify their pod-specificity and relative expression. These clones were named POD1, POD2… POD30. A series of 30 northern hybridizations were done with these clones. Total RNA from different organs of peanut resolved on agarose gels were hybridized to ^{32}P-labeled POD clones inserts. Of the 30 clones analyzed, only POD3 exhibited strong expression in pods

and not in seeds (Fig. 4A) and was chosen for further analysis. Figure 4B shows a control hybridization using the same RNA as was used in Fig. 4A hybridization, with spinach 28S rDNA probe. All lanes contained 30 μg of total RNA measured by OD_{260}. Some expression, albeit weaker, is seen in seed coat. The bands were scanned, and their inverted images were analyzed for intensity. The ratio of the POD3 hybridization signal intensity to that of 28S was 0.30 for pod RNA and 0.1 for seed coat RNA. The seed coat signal was only 10% above the background and the accuracy of this estimation may be limited by background variability and by the X-ray film response curve. These images were not analyzed by PhosphoImager. The seed coat expresion could be because of some promoter activity in seed coat, or because of cross-contamination of the tissues. Cross-contamination cannot be ruled out due to the fact that pod and seed coat share the vasculature at the vine and during separation the vessels could be attached to either tissue. Ribosomal RNAs are the major RNA species in all cells and thus can serve as a control of RNA integrity and loading. All other clones either did not show a hybridization signal with peanut RNA or hybridized to peanut RNA from multiple organs. Among those, we had cDNAs that hybridized to seeds more than to pods, and those that hybridized to RNA from all tissues used for RNA analysis, that is, to RNA from seed coat, leaves, pods and seeds. These clones were not used for further experiments in this project.

To determine whether our gene of interest, POD3, may be a part of a gene family, or may be present in more than one copy because cultuvated peanut is an allotetraploid, gene copy number was determined. This was important because the genes from small gene families were considered better candidates for finding the genomic clone because the genes that are members of large gene families would require larger number of positive

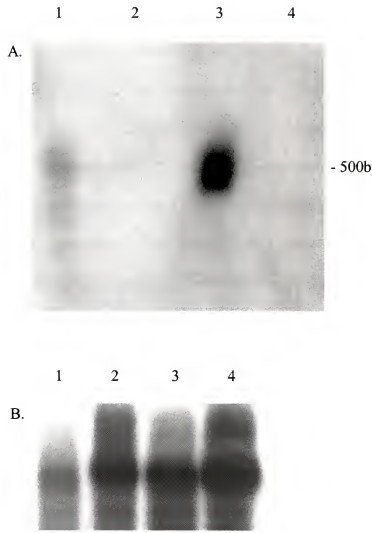


Figure 4. Peanut RNA hybridization showing that, of the tissues evaluated, POD3 is expressed in pods but not in seeds. Total RNA from the following tissues is blotted onto the hybridization membrane: 1 - seed coat, 2 – leaf, 3 – pod and 4 – seeds. A. Hybridization with POD3 probe. POD3 mRNA size measured by RNA marker is shown. B. A control hybridization with the spinach 28S rDNA probe with the same RNA loading as in A.

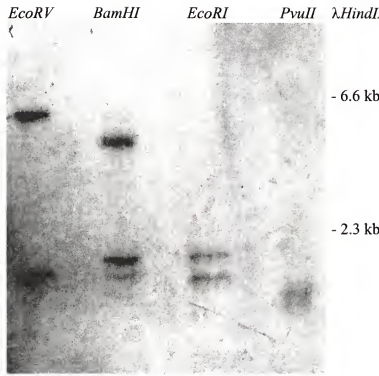


Figure 5. Southern blot of peanut genomic DNA hybridized with POD3 cDNA. DNA was digested with the restriction enzymes indicated. λ*HindIII* = *HindIII*-digested λ phage marker sizes.

genomic clones to be isolated in order to find one that was expressed in pods and detected by Northern analysis. In order to estimate the POD3 gene family size, a Southern hybridization was done. Peanut DNA digested with *EcoRV, BamHI, EcoRI and PvuII* restriction enzymes was hybridized to POD3 probe (Fig. 5). None of the restriction enzymes used had a recognition sequence in the POD3 probe, and all of them gave two hybridizing fragments of similar intensity ranging 1-6 kb in size. This suggests that POD3 is not a single-copy gene and that it most likely consist of two members.

Cloning and Characterizing POD3 Genomic Clone

To obtain the regulatory sequences of POD3, a peanut genomic clone of this gene together with sufficiently long upstream region containing pod-specific expression element(s), was necessary. A peanut genomic library was not available to us, so a partial genomic library was made with the aim of isolating the POD3 genomic DNA. To design the strategy, Southern analyses of genomic peanut DNA cut with restriction enzymes were hybridized with POD3. Some enzymes either produced bands of the size which exceeded a λ cloning vector insert capacity (12 kb), or produced bands that would be too small to contain the entire POD3 gene and its regulatory region. The completeness of genomic DNA digestion was not checked. Some enzymes did not appear to cut genomic DNA at all, possibly due to methylation sensitivity or the enzyme or DNA quality. So, the banding pattern of hybridization seen in Fig. 5 was not reproduced. However, the experiment served its purpose and a suitable enzyme was found. *XbaI*-digested DNA gave a single strong band hybridizing to POD3 of 7 kb. Therefore, the *XbaI* fragment library was chosen for cloning.

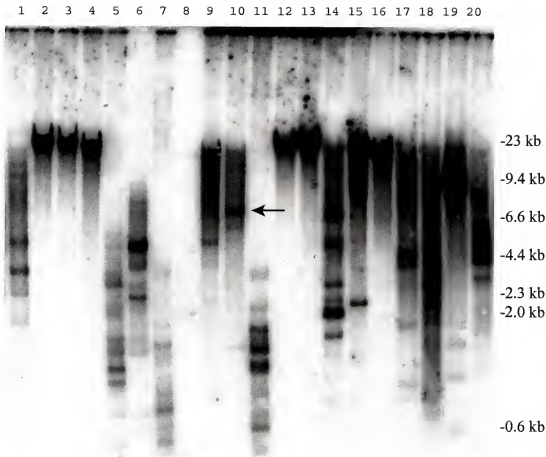


Figure 6. Screening of restriction enzymes for an enzyme producing a single high molecular weight band of peanut genomic DNA hybridizing to POD3. An arrow points to the XbaI-digested DNA band of 7 kb, which hybridized to POD3. 1=*BamHI*, 2=*SalI*, 3=*PstI*, 4=*PvuII*, 5=*HincII*, 6=*DraI*, 7=*Sau3AI*, 8=*HindIII*-digested λ phage marker, 9=*ScaI*, 10=*XbaI*, 11=*RsaI*, 12=*ClaI*, 13=*SmaI*, 14=*Bsu36I*, 15=*KpnI*, 16=*ApaI*, 17=*EcoRV*, 18=*MspI*, 19=*NdeI*, 20=*SacI*. The DNA marker band sizes are indicated.

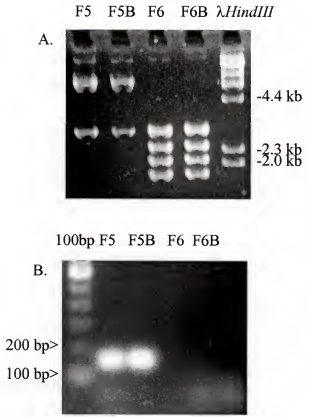


Figure 7. Analysis of the clones excised from the positive plaques. A. Plasmids digested with *XbaI*. F5 and F5B produced a 3 kb vector band and a 7 kb insert band, whereas other clones did not produce the 7 kb fragment. λ*HindIII* = *HindIII*-digested λ phage DNA fragments.The sizes of the fragments are indicated. B. Plasmid PCR amplification with POD3-specific primers RES53 and RES58 (see Material and Methods). 100bp = 100bp DNA size marker. The sizes of marker bands are indicated. F5 and F5B produced a fragment of predicted size, 163 bp.

The size of hybridizing fragment(s) should be large enough to contain the upstream POD3 regulatory sequences. Lambda ZAP II vector (Stratagene) was chosen for cloning because it contains an site in the polylinker, is capable of accepting DNA fragments of 0-10kb and allows for the blue-white selection of clones with inserts. The partial library consisted of genomic DNA fragments ranging from 6-9 kb. The primary library titer was $3.5x10^5$ pfu and, when plated on X-Gal and IPTG, about 70% of the plaques were white, suggesting effective ligation of inserts. The primary partial library was amplified and approximately $3x10^5$ plaques were screened by three rounds of hybridization with POD3 probe. Two positive plaques, F5 and F6, were isolated and their inserts were *in vivo* excised as pBluescript SK(-) plasmids. Two colonies were picked for plasmid DNA isolation from each excision plate. F5 and F5B were picked from a plate containing excision products of F5, whereas F6 and F6B were picked from a plate with F6. Figure 7A shows the clones digested with . Only F5 and F5B had the insert in the vector that measured 7 kb, the size of the fragment hybridized to POD3 (see Fig. 6). Figure 7B shows the PCR products of these clones amplified with POD3-specific primers RES53 and RES58. Only F5 and F5B produced a band of 163 bp which corresponded to the distance between these primers in POD3 cDNA. The correct size of the PCR product using POD3-specific primers indicated the presence of POD3 sequence in F5, and the insert was sufficiently large to expect the presence of upstream POD3 sequences in this clone as well. Based on these data, F5 was selected to be sequenced as the genomic clone corresponding to POD3.

```
   1  ACAACA AGAATAACAG GAGAACTTAG AACAACAACG

  37  TAGAAGAACA GTGTAACAAA GAAGCAAGAA TAACAGTAAA TCCTAGAACA

  87  ACGACGTAGA AGAAAAGTAA AACCTAGTTC GTAATCTGAA AAATATACAG

 137  GAATCTTAAT GAACTTACCT TGAGTATTGT TGCTTTGTTT TCTCTTCAGA

 187  TTCTTGATGG AGAGTTTGAT GGTGTGTTGA TGGAGGTTTC GAAGGTTAGC

 237  GACGACTTGT ATATTTTGAA CTTCGATTTT CGCTCGAAAA TGGAAAGATT

 287  GCTTTGTTTT CGAATCGCTT TGAGAAGTGG AAGAAGTGGA AGAGTCTGCC

 337  ATCACTCGTA GCGTGCGTAA CCGTTTGAGT AGCGAGCGCG TGAATGTGAC

 387  GCTCCATTCA ATCCGTCTTC ATGCGCGTAA GTTGTATTTG GGCTGGGTCA

 437  ACTTGTAAAC TTGTAAACTT GTATGTGTAG CAGACCCGAA AAAAGTTAAT

 487  AAACACACAA TCTTTTTGTT TGTGATTATA ATTAGAAACT TACTACTCTC

 537  TCTCTACTAT ATTTTAATTA ATAATATTAT CATACAAAAA AGACTTTTTT

 587  TTAAATAGAA TCTAAATAAA GTAATCACTA CAAGAGATTT GATTATTTAA

 637  CTATAAAAAG TTTGATTATT TTACAGTAAA ATATTTGATT ATTCTAATAA

 687  TTGATTTTTT ATTAAAAAAT ATAAAATAAT ATGTATAAAT ATATATAAAT

 737  ATTCAATTAC TAATTTTTTA TGAGTGATTT TTGGTGTTTG TAGAATATTT

 787  ATCTTCAGAA TTTTTTTCAT CGAACTGTCA AAATATAGAT TATTATTATT

 837  ATTATTATTA TTATTATTAT TATTATTATA TGAAAAAGAG AAATATAATT

 887  ATATAAATAT ATAATTATTA AAATAAGTTG AATCTTTCCG ATTTTTCACC

 937  TTAATCTCAA ATTGAGTTGT TTTTTCCAAT TATATAAAAA TGATGAAATT

 987  GTAAAATTAG TATTTTAAAA ATAAAAGAAG AAATGCAGGC AGAGAGATTC

1037  CTACAGATAA AAAATTCTCA AGTGATGGGA GACGTATACG AATACGACCC

1087  ACATGGGTTG TGAGGTAGTC CAATGAATAA CGCATACTAC TACTAGATAG

          TATA      TATA                                +1    +1
1137  GTCTTTATAC GGCTATAAAT AGTGGATCAT GTTTGAAGCC TTGGGAGGCA
```

Figure 8. The partial sequence of F5 from the 5' end of the genomic clone to the 3' end of POD3 cDNA. The PCR and hybridization primer sequences are shown. Predicted TATA boxes and poly(A) addition signal are indicated. Transcription start sites are indicated by +1. The initiation and termination codons are marked by **M** and *, respectively. The sequence of the differential display cDNA is underlined. The cDNA sequence obtained by 5' RACE is bolded. The POD3 intron sequences are in lowercase letters.

```
     5'AGCAGCATA CATAGCATT 3'-> RTPOD3F
1187  TAGCAGCATA CATAGCATTA ATTTATTTGC ATTCCACAGA TAATTAAGTC

              M      5' ACCTG CGGAAACTGC GACTGCG 3'-> PODHYB
1237  ATCAAAATGT CGAACACCTG CGGAAACTGC GACTGCGCTG ACAAAACCCA
                <- 3'GTGGAC GCCTTTGACG C 5' POD3-4d
        <- 3'ACA GCTTGTGGAC GCCTAGGA 5' POD3-5d

1287  GTGCGTgtaa gtccttcact tctatttgca tctatctact tctccattac

1337  aaatatgctt tttcatatct ccttcaaaat ataattcctt tatgcaacat

1387  tctccaaaat tattgctaaa agacttagct tattaggcta gaaattatta

1437  ttacacttgt taaaagccag atttttccaa ccacgcttgt ctaggacaaa

1487  tatgttttgt acacattcaa tttgaataaa aatatgttga ctaaattatt

1537  gtttttgtct ctaacatttg gggtaagtct tatttgtgtt tctaacgttt

1587  aaatcgtctt atttgtatcc ttaacgttta taaaagtgat tcaatgttat

1637  cctactatca attatactaa cagatcagat tatatttttc aattattctc

1687  acttagatgt attcattctc aattaggttt tacttggatg tgttcaattt

1737  taatattata cccactattt atatttagat tcaattatct ccctaaaaaa

1787  gtgaattacg taaatgttat agaaattagt ttcaactttt gatgagctat

1837  ttttcaaagt gaatcatcaa ttctattcta taaatttgta ttctaacttc

1887  aagaataaat ttttaaaact caaactaaag cattcatgat gtgtaattaa

1937  tggcaggata atattgaatc acttttacaa acattaagga tacaaatagg

1987  acgatttaaa tgttagagat acaaatagaa tttaccctaa atattggaga

2037  caaaaacgat attttattca aatatattct tttgtattca ccgtcctcca

2087  tgaagaaaaa aaaaaactgt ttatcaattt gtacaagtat ttgctttttct

2137  tccaaataag aagaaagcat gaagaaactg agaaagagga gaaaaagtt

2187  aaatgaagag cataagaaaa acataattaa aaaaattaac aactaattta

2237  gtatcgaaaa gatttaaatc tttgattgat ttaaaaatgt gataagattt

2287  ttttattttt taaaaaaatt tgatcattca cgtaataaat ttttaaaaag

2337  attcatttaa cataaaattg tcaaatttta aagatgaaaa ttttattttt

2387  ttaataatac ttacaaaaaa ttacatcaaa atctaaacta taaaattagt
```

Figure 8 -- continued

```
2437  ttttaaaata tatatttaat atctgatttt tttgtcaatt ttttaatttt

2487  tcgaaaactt atttattgtt agagtttttt ggaattattt ttgtgaccga

2537  tgtgaacttt taagtattat tttagtaatt tattcttaaa aaaataaaat

2587  gaagaatggc aaacaaaagt aagaatgaag ggtaaaaaaa ataaaaatta

2637  acgcacgcat gcttcttgaa atgtcttgtg aactccagta gcaaaatcca

2687  attcttacca gactacacta tttgcatacg gagtagtatt ggaaggtaat

2737  atctaatacg atcaaaactc taataaaata taataatatt tggtgttaac

2787  tatagaaaat tctagtacgc tactaacaca tttcagtatt tcacataaaa

2837  atattggtta aatccaaata tacgttgtac aaatttggaa taataaaatt

2887  cctaaagatg agaagaatat gtttaaattt aaattataat acatcaaatt

2937  ttatatccta ttgactaact tgattctcga cgcaaagaac cttatgtgtg

2987  tttaaattgg agtagtatac ttagcttgca aatagagtgc gcagaagaaa

3037  aaaaaaattg atactagaca gttttctatt attaaaaata taaattcaaa

3087  aatagctttt tgaaaaaata taattatctc aaaataaaat catttcataa

3137  atatcataat ttaattttgt gctatatgcc taataataat attttgatac

                                        WH908 5' GT TCAGGAAGGG
3187  ttggatttac aaagataact cattctttca ttatttatgt tcagGAAGGG
                            WH909 (cont.) <- 3' CA AGTCCTTCCC

      ATCCAAATAC GGT 3' -> WH908 (cont.)
3237  AAACAAATAC GGTGTTGACA TCGTGGAAAC CGAAAAAAGg taacctctac
      TAGGTTTATG CCACAAC 5' WH909
               <- 3' AACTGT AGCACCTTTG 5' RTPOD3R

3287  attttggcaa tttctaattt ttctttaatt tgtaaacaaa tactgaccta

3337  gtgtcctatt ctctaatttt ttaaataaaa tattattttt attcctaaca

3387  tttggagtat attttaaaat tatttctatc atttaaattg tccaatttaa

3437  aattgtgtcg acattatcct accattagtg atttgttaat gaaattgacg

3487  gtaagataaa attgagataa ttttaaaatg ttagggacaa cttattcttt

3537  actttctttt attaagtaat gtttttgttt tatttaagtc ttaacgtttt

3587  aaaatcgttt caattttttt taccatcaat tatgttagca gattcctaac

3637  gacaaaacaa tattaaacaa attttaaaat attaaagatt taaataggac

3687  gatttaaacg ttaactgacc acaaacatta aagataaaaa ctatatattt
```

Figure 8 -- continued

```
3737  tttttacact tttacttcct actatttctg agaataccaa ttatatttgg

3787  tttcaataat ttttttaatc tccgatctaa tgattgtctt actcgtgatg

3837  caaatccaga aagctaaata caataattac acgggaaagc ttattaaaat

3887  tgcaatatat atgtttttt acagGATGGT GGAGACTGTG GTGATGGAAG
    RES70 <-3' A TACAAAAAAA TGTCCTAGGT AA 5'   POD3->

                                        RES53 5'TGCGGTGC TAACTGCTC 3'->
3937  TTCCAGCTGG TGAGAACGAT GGGAAGTGCA AGTGCGGTGC TAACTGCTCT
          <- 3'ACTCTTGCTA CCCTTCA 5' POD3-1d

3987  TGCACCAACT GCACCTGTGG CCATTAAGTA TAATGTAATT GCCAAATCTG

                                          poly(A)
4037  TTTGGCAATA ATAATATGTA CTCTTTTACT AAATAAAACT GTGTGCTGTT

                          *
4087  GTCGTATTGC ATATCTGTGT GAGTAAAATG CACTCTACGT ATTTGAATCA
                  <-3'GACACA CTCATTTTAC GTGA5' RES58

4137  AGTTTCGTTC ACAGTGATAG CTAGCTATAT AACAGCCATG GCTCTGCCAT
            <-POD3

4187  TTTATGGTTA CATTTATGGA CTATAATATG CATATCTCTT GCTAGTTGCT

4237  TCCTACCTTA GCTTCATGTC TCAGTTATTA TCATGAACAT CAAATTTTAT

4287  ATATACACTT AAACTCCAAC ACCAACAACC GTAATAAAAA ATGAATTTTG
```

Figure 8 -- continued

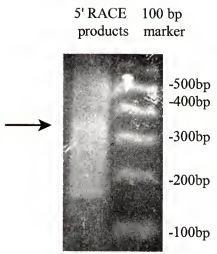


Figure 9. Agarose gel of 5' RACE products obtained with POD3-1d reverse gene-specific primer. The left lane contains the PCR product of the 5'RACE reaction (marked by an arrow), and the right lane has a 100 bp marker with the sizes indicated.

Generally, gene regulatory elements lie upstream of a coding region. Therefore, the portion of F5 immediately downstream of POD3 cDNA, the region homologous to the cDNA and the entire region upstream of it were sequenced in both directions by primer walk (Fig. 8). Double stranded plasmid DNA was used to sequence all regions except a region of F5 spanning nucleotide positions 400-1000 in reverse direction. In this region there is a stretch of 97 bp containing only three guanosine residues and no cytosines, and, apparently, this very AT-rich upstream regulatory region of the gene interfered with sequencing reaction. To overcome this difficulty, single-stranded DNA of F5 was produced and sequenced. This template DNA gave over 750 nt of the readable sequence in the difficult region. The sequence of F5 between nucleotides 3924 and 4148 (Fig. 8) was identical to POD3, which confirmed that F5 is a genomic clone of POD3.

Isolation of the 5'- end of POD3 cDNA.

The differential display technique does not produce full-length cDNAs. It was not known to us where the POD3 mRNA transcription starts, where its promoter is and whether the gene has any introns. To address these questions, a 5'-end of the POD3 cDNA was cloned by using the 5' RACE technique. To conduct the 5' RACE experiment, a Marathon™ cDNA amplification kit (Clontech) was used on peanut RNA from pods at stage 2. Initial attempts to amplify the 5'-end of POD3 using RES58 as a gene-specific reverse primer resulted in a 5'-end of cDNA identical to that of POD3. When RES58 was used for primer extension, the reaction products all had the 5'-ends only a few nucleotides upstream of 5-end of POD3. It was hypothesized that something in the secondary structure of the POD3 mRNA prevented first-strand cDNA synthesis using the RES58.

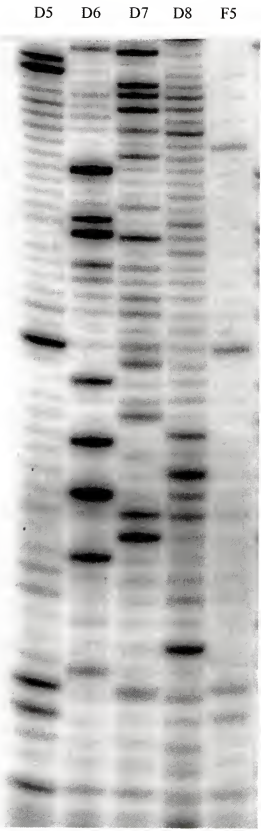


Figure 10. Screening of 5' RACE products using a sequencing reaction. A gel fragment containing the products of the sequencing reactions with dideoxyGTP terminator is shown. Lanes contain different DNA templates. D5, D6, D7 and D8 are 5' RACE clones. F5 is POD3 genomic clone (control). Marked by '<' are the G nucleotides that are in the same positions in D5 and F5. D5 and F5 begin to differ towards the top of the gel fragment suggesting an intron. Twenty-nine other clones did not have the same G nucleotides next to the primer and are mispriming artifacts.

Therefore, another attempt was made to synthesize the 5'- end of POD3 cDNA from a primer binding at a site upstream of RES58 and very close to the 5'-end of our initial differential display clone (nucleotide positions 3947-3963, see Fig. 8). This primer, POD3-1d, extended further upstream, and, after completion of the 5' RACE protocol, we had a band of 340 bp (Fig. 9). The 5' RACE fragments were cloned and 30 clones with inserts were screened by sequencing primed by POD3-1d, and containing one of the four dideoxy-terminator nucleotides. The sequencing reaction with F5 as a template with the same terminator nucleotide was run on an adjacent lane of the sequencing gel. The weak bands of the sequencing ladder in Fig. 10 represent the premature termination products and the stronger bands, marked by '<', represent guanosine residues. Only one clone, named D5, had guanosines at its 3' end at the same positions as F5 and was chosen for further analysis as a cDNA most likely corresponding to F5 genomic clone.

The POD3 cDNA Sequence Analysis.

The POD3 cDNA sequence was derived by aligning the sequence of the differential display clone POD3 and the sequence of the 5' RACE clone D5 in the overlapping region. POD3 contains an ORF of a metallothionein-like gene 195 bp long with a stop codon TAA upstream of the first ATG triplet, indicating that this is the true initiation codon (see Fig. 8). This ORF was used to search the non-redundant Genbank peptide database and was found to be most similar to the type II metallothionein-like genes. Figure 11 shows the 65 amino acid sequence alignment of the translated POD3 ORF and a published metallothionein-like gene from papaya (*Carica papaya*). Note the

perfect alignment of conserved cysteines essential for metallothionein function (Robinson et al., 1993).

POD3 Gene Structure.

The alignment of the POD3 genomic clone and cDNA sequence has revealed its gene structure. The gene consists of three short exons of 114 bp (1179-1292), 45 bp (3231-3275) and 238 bp (3911-4148) separated by two relatively large introns of 1938 bp and 635 bp (Fig. 8). Both introns start with GT and end with the AG dinucleotide in accordance with the GT-AG rule for introns (Brown et al., 1996). The 3'-portion of POD3 contains a polyadenylation signal consensus sequence (Lewin, 1994).

```
POD3 (peanut)     1 MSNTC GNCDCADKTQCV.KGNKYGVDIVETEKRMVETVVMEVPAGENDGK 49
                    ||.||||||||||||| ||. |  ||:|||| .. ||||: || |||||
Y08322 (papaya)   1 MSDTC GNCDCADKTQCVKKGSSYTADIIETEKSIM.TVVMDAPAAENDGK 49
                      .
POD3 (peanut)    50 C KCGANCSCTNCTCGH 65
                       |||| .||||||||||
Y08322 (papaya)  50 C KCGPSCSCTNCTCGH 65
```

Figure 11. The alignment of POD3 amino acid translation and a protein which has the most similar sequence among all published proteins, a metallothionein-like protein from papaya, found by BLAST program. A Genbank accession number for the papaya mRNA sequence is given. Vertical bars show identical amino acids. Dots show similar amino acids, and two dots represent greater degree of similarity than one dot. The conserved cysteines (C) are underlined.

Two Transcription Start Sites as Determined by Primer Extension Experiments.

As stated earlier, our first primer, RES58, failed to extend to the 5'-end of POD3 cDNA. As with 5' RACE (above), we obtained better results with a primer POD3-1d farther upstream. It produced doublet of primer extension products about 200 nt long suggesting there are two RNAs differing by 5 nt in length as measured by an F5

sequencing ladder. At that time we could not precisely map the 5'-ends of these primer extension products because of their relatively large size and because the sequencing ladder had a nucleotide composition different from that of the first strand of the cDNA (Fig. 12). Note that the higher molecular weight artifact bands in the pod mRNA lanes disappeared when reaction was carried out at higher temperature. This suggested that the primers at lower temperatures annealed and extended in the wrong places with some mismatch. In order to confirm the transcription start positions, a different primer, named POD3-4d, was designed for primer extension closer to the predicted transcription start sites. It also gave a doublet of bands at the same positions as the POD3-1d primer, but since the extension products were shorter and there were no introns between the primer and the transcription start sites, we were able to determine the exact nucleotide position of both transcription start sites (Fig. 13). Each of the POD3 RNA species produced a single band at 45^0C, but at 42^0C, bands that were 1 nt larger than the bands obtained at 45^0C were also visible. These upper bands are likely to result from 'hook' structures that reverse transcriptases can form (Sambrook et al., 1989), so the transcription start sites were mapped to nucleotides corresponding to the single bands obtained at 45^0C (marked by arrows). Multiple transcription start sites have been reported (Wilhelm et al., 2001; Liu et al., 2001). Another explanation for two bands 5 nt apart on the gel would be a stalling of the reverse transcriptase because of the secondary structure. This explanation seems unlikely because the temperature of the extension reactions did not affect the intensities of the bands with respect to each other. Putative TATA boxes are indicated on the sequence (Fig. 8) upstream of transcription start sites. They are positioned 28 bp and 34 bp upstream of the first and the second initiation sites, respectively, and these

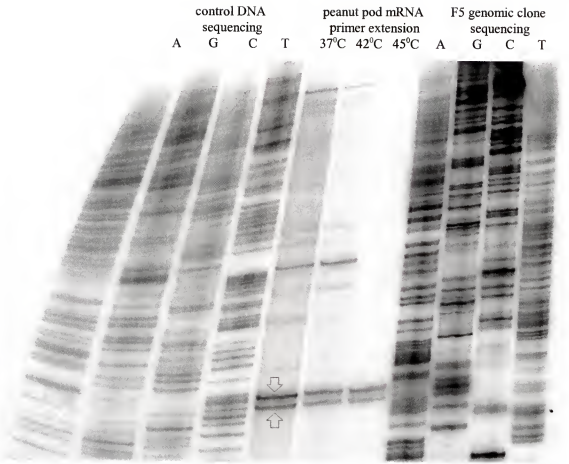


Figure 12. POD3-1d primer extension PAGE. Control DNA of pUC19 was sequenced with universal sequencing primer. Both primer extension reactions on pod mRNA and F5 sequencing reactions used POD3-1d as a primer. The temperatures of primer extension reactions are indicated. The primer extension products are indicated by open arrows.

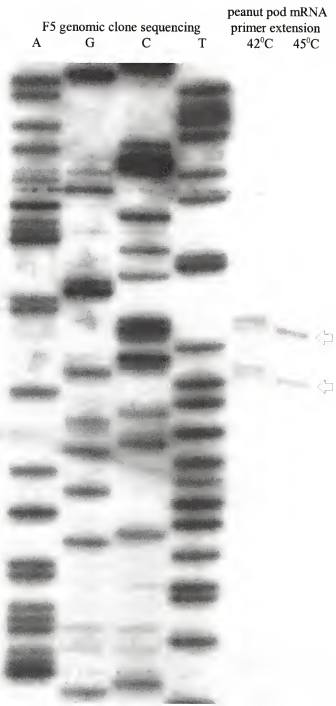


Figure 13. POD3-4d primer extension PAGE. Both primer extension reactions on pod mRNA and F5 sequencing reactions used POD3-4d as a primer. The temperatures of primer extension reactions are indicated. The primer extension products are indicated by open arrows.

distances are closer to those most common for TATA-boxes. The second TATA also has AATA sequence downstream of it, and in eukaryotic promoters, TATA is generally followed by a 4-mer consisting of adenosine and thymine residues (Lewin, 1994).

Cloning of POD3-GUS Constructs.

The POD3 genomic clone includes 1180 bp upstream of the POD3. Promoters usually contain all proximal regulatory elements within 110 bp upstream of transcription start site, and many trans-activating sequences lie within several hundred base pair region around the transcription start site. The majority of regulatory sequences are found upstream of the transcription start site, but some are found downstream of it (Clancy et al., 1994). In order to map the regulatory elements of POD3 gene, we cloned POD3-GUS fusion expression constructs. Three translational fusions of POD3 genomic sequence to the GUS gene sequence with a transcriptional terminator from the nopaline synthase gene (NOS) (Jefferson et al., 1986) were cloned in pUC19. They were named PODGUS1, PG1 and B5 and they had the upstream genomic sequence of POD3 fused in frame to the GUS coding region in the first, second and third exon of POD3, respectively (see Fig. 1 and 2).

Analysis of Gene Expression in Transgenic Alfalfa.

The longest POD3-GUS fusion construct, B5 containing both introns, was inserted into the pBI121 binary T-DNA vector purchased from Clontech, and the resulting construct was used for *Agrobacterium tumefaciens*-mediated transformation of alfalfa cv. Salomac. Fourteen plants were regenerated on selective media containing kanamycin and analyzed for expression of NPTII, the kanamycin resistance gene. Young leaves of regenerated plants were analyzed for NPTII expression to verify transformation. In addition to being a proof of plant transformation, it also showed the relative NPTII

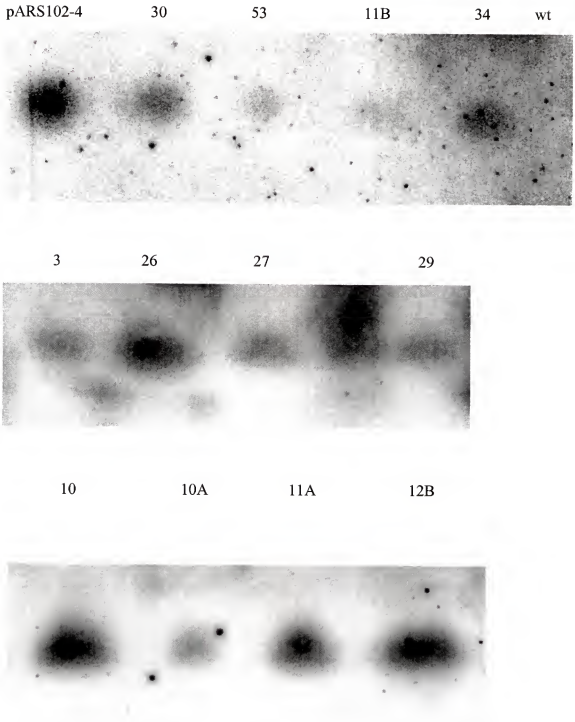


Figure 14.
The NPT-II analyses of the crude leaf extracts of transgenic alfalfa. pARS102-4 is a positive control plant and wt is an untransformed plant. All others are different plants transformed with B5.

pARS102

wild type

10A

26

30

Figure 15. X-Gluc stained alfalfa leaves. Plants 10A, 26 and 30 were transformed with B5. pARS102 was a positive control.

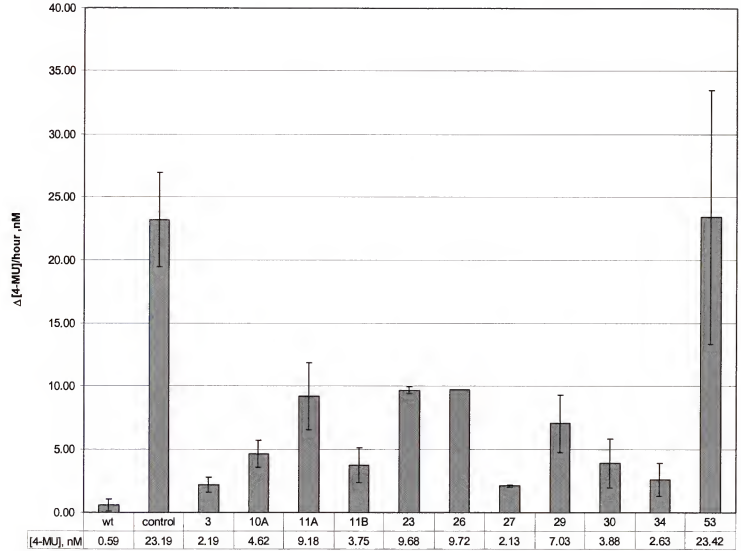
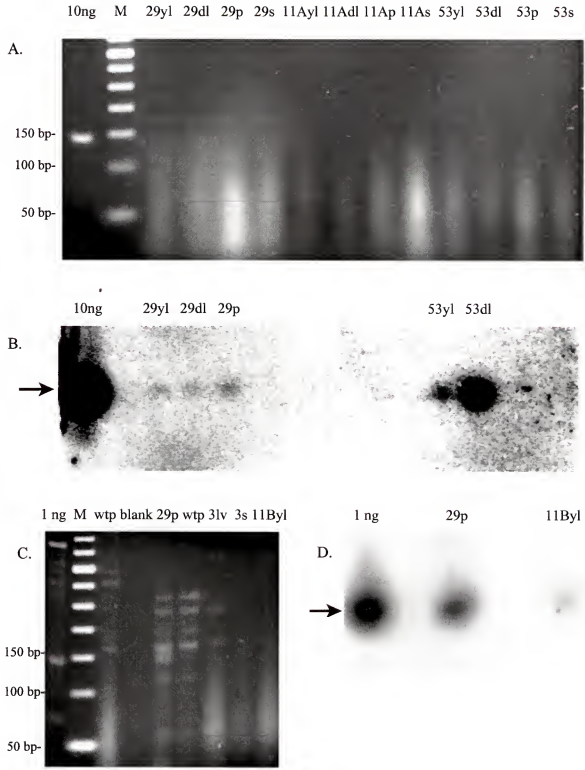


Figure 16. The MUG assay data on alfalfa leaf extracts. Numbers on the horizontal axis represent different transformants. wt - untransformed plant, control - a positive control plant pARS102, all others are transformed with B5 (POD3-GUS). Error bars represent standard deviation of mean of a series of two to four measurements.

Figure 17. The analysis of the POD3-GUS chimeric mRNA in transgenic alfalfa plants. Panels A and C are photographs of ethidium bromide stained gels of RT-PCR products, and panels B and D are the results of hybridizations of DNA transferred from gels shown in panels A and C, respectively, with ^{32}P-labeled probe PODHYB. Only the lanes which produced a hybridization signal are labeled in panels B and D. The 141 bp hybridizing fragments are indicated by arrows in B and D. The gel lanes are labeled as followed: 1 ng, 10 ng=amount of the peanut pod RNA used for a positive control RT-PCR, M=50 bp ladder. DNA marker sizes are indicated. All other lanes are labeled with transgenic plant number and tissue type used as templates. wtp = untransformed pod RNA. The tissues used were: yl=young leaves, dl=dark leaves, l=leaves, p=pods, s=seeds.


10ng M 29yl 29dl 29p 29s 11Ayl 11Adl 11Ap 11As 53yl 53dl 53p 53s
A.
150 bp-
100 bp-
50 bp-
10ng 29yl 29dl 29p
53yl 53dl
B.
1 ng M wtp blank 29p wtp 3lv 3s 11Byl
C.
150 bp-
100 bp-
50 bp-
1 ng 29p 11Byl
D.

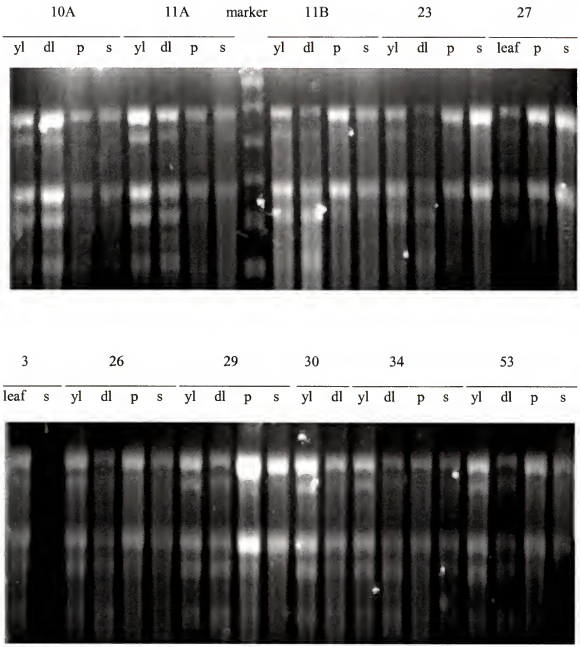


Figure 18. Ethidium bromide stained agarose gel of RNAs used for RT-PCR. Each lane contains 4μg, as measured spectrophotometrically, of total RNA from leaves (leaf), young leaves (yl), dark leaves (dl), pods (p) or seeds (s) of indicated alfalfa plants.

expression levels in different transformants (Fig. 14). The same volume (50μl) of the crude leaf extract was loaded to analyze each plant. The detectable NPTII expression suggested that the T-DNA integrated in a transcriptionally active euchromatin site. Thus, if the NPTII gene is expressed, one would expect the other inserted gene, β-glucuronidase, might also be expressed. The promoter activity of the B5 construct was also evaluated by *in situ* staining of the pod, seed and leaf tissue samples in the staining solution of a chromogenic GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). Initial experiments on the GUS expressing positive control plant pARS102 (Shatters et al., 1998) expressing GUS under the control of a modified CaMV 35S promoter and on untransformed alfalfa revealed that seeds and pods of both the positive control and untransformed plants stained blue making this assay of little value for analyzing GUS expression in those tissues. The leaves of untransformed alfalfa showed no blue color, whereas GUS-positive pARS102 leaves were blue. Therefore, only leaves of plants transformed with B5 construct were analyzed. Not all plants were analyzed, and only the leaves of three plants which stained blue are shown (Fig. 15). It appears that in alfalfa leaves, the B5 promoter is active late in development, suggesting some specificity. However, the small number of plants analyzed *in situ* together with known variability in expression of genes introduced by *Agrobacterium* does not allow us to draw firm conclusions about B5 tissue specificity in alfalfa. In order to quantify GUS activity in the leaves of transgenic plants, GUS activity was measured in alfalfa leaf crude extracts. The seed coat of alfalfa was not analyzed at this time because of its small size. The 4-methylumbelliferyl-β-D-glucuronide (MUG) assays were conducted two to four times on crude extracts from alfalfa leaves as described in Materials and Methods. Figure

16 summarizes these results. The increase in 4-MU concentration, in nanomoles per liter, after 1-hour incubation of the leaf extracts in a reaction buffer with MUG and subsequent dilution in 0.2 M Na_2CO_3 is plotted on the vertical axis. The GUS-like activity of the extracts from untransformed plants (wt bar on Fig. 16) was lower than that of any of the transformed plants analyzed. The GUS activity levels in B5-transformed plants varied, but none of them had GUS activity higher than a positive pARS102 control plant.

Because the GUS-like endogenous activity of alfalfa seeds and pods prevented GUS analysis of B5 promoter activity in those tissues, attempts were made to measure mRNA. First attempts to detect the POD3-GUS transgene mRNA transcribed from the B5 construct introduced in alfalfa by northern hybridization were not successful. Apparently, that RNA expression level is below the detection limit of this technique. Next, reverse transcription followed by PCR (RT-PCR) was tried. The binding sites for RT-PCR primers, named RTPOD3F and RTPOD3R, were chosen in exons 1 and 2, respectively (see Fig. 8), so that only the correctly spliced mRNA will give rise to 141 bp long PCR fragment, whereas both B5 DNA and unspliced B5 transcript will produce a 2.1 kbp long PCR fragment. The primers easily amplified 1 ng of the total RNA isolated from peanut pods harvested at the stage 3 (Fig. 17A), suggesting high transcript abundance. RT-PCR reaction was optimized by varying the following PCR parameters: $MgCl_2$ concentration, number of cycles and the annealing temperature. The goal of optimization was to find the conditions when the difference in amount of newly synthesized DNA is greatest between the reactions with RNA template from the untransformed (wt) plant and plants transformed with B5 that have good expression in GUS enzyme assays. Plant 29 was chosen because of its high GUS activity in leaves (see

Fig. 16). Besides the amplified fragment of 141 bp, the optimized reaction still produced additional bands with RNA template from both untransformed and transformed plants. Some of them had sizes close to 141 bp, which made it difficult to prove that the band of correct size had the right sequence, that is, originated from the introduced gene. A 141bp product was not visible on the ethidium bromide stained agarose gels when RNA from other plants was a template. To increase the sensitivity and specificity of RT-PCR, Southern analyses of those reaction products with a ^{32}P-labeled probe PODHYB (see Fig. 8) with a binding site between the amplification primers was carried out. The results of these hybridizations are presented in Figure 17. The input RT-PCR RNA samples were electrophoresed on a denaturing RNA gel and stained with EtBr to confirm the integrity of template RNA (Figure 18). RT-PCR followed by hybridization was more sensitive than RT-PCR but could not be used to derive quantitative results. Not all plants having GUS enzyme activity contained a detectable amount of the transgene mRNA. This may be due to the very low expression level of B5 in alfalfa or due to relative instability of its mRNA.

The results of experiments on alfalfa transformed with B5 promoter construct are qualitative rather than quantitative due to low number of plants analyzed and because not all plants were analyzed by all methods. In the alfalfa heterologous expression system, B5 version of POD3 promoter was not pod-specific. In leaves, GUS accumulated at a level comparable with that in a positive 35S-GUS control plant (Figs. 15 and 16). There also was a single plant in which B5 mRNA was detected in pods (plant 29, see Fig. 17). In summary, the POD3 promoter activity in alfalfa suggested testing its truncated versions in an expression system in search for the regulatory elements of transcription.

Transient Promoter Expression of the Fusion Constructs in Tobacco Suspension Cells.

Tobacco protoplasts are a commonly used gene expression system in which to study plant promoters expresing GUS reporter because of a low endogenous GUS-like enzyme activity (Czarnecka-Verner et al., 2000). A promoterless GUS construct NOPGUS, PODGUS1, PG1, B5 and a positive control CaMV35S-GUS construct pBI221 (Clontech) were introduced into tobacco protoplasts by PEG-mediated transformation followed by the transient GUS expression assay (Fig. 19). The data within each chart were produced from homogenized protoplasts from the same tube. Each time, the protoplasts are different in the efficiency of transformation, viability and ability to express reporter genes from different promoters (Dr. Eva Charnecka-Verner, personal communication). Therefore, one can only compare promoters of different constructs if they are transiently expressed in the protoplasts from the same preparation. In spite of that, these experiments allow us to quantitavely compare the constructs withn the same protoplasts. The POD3 promoter constructs with one and two introns, PG1 and B5, had the least active promoters and were not significantly different with respect to each other ($p>0.05$ in all experiments). In comparison to them, PODGUS1 is approximately 6-fold more active in this expression system. PODGUS1 construct expressed GUS at a similar or higher level than pBI221, where GUS gene is driven by CaMV 35S, a strong dicot promoter (Sanders et al., 1987). The ratio of the GUS activity expressed by PODGUS1 versus pBI221 varied from 1.5 to 2.7 between different protoplasts (see beginning of this paragraph for a possible explanation), so one can only say that this version of POD3 promoter is at least as active as 35S in this expression system.

Clearly, this set of data showed that PODGUS1, the promoter construct without

A.

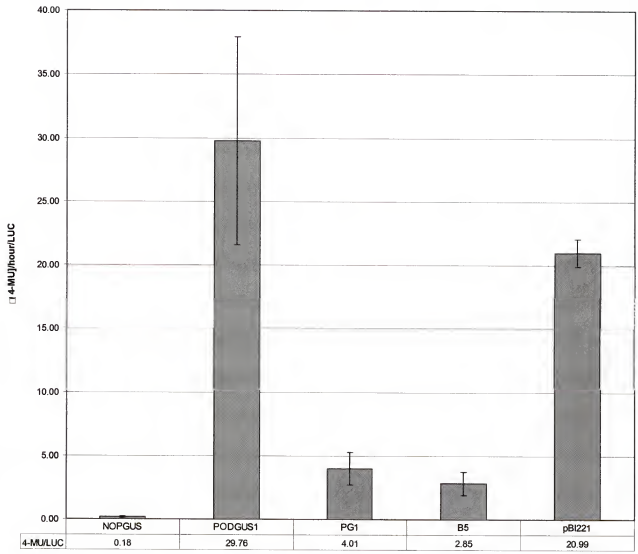


Figure 19. The averaged results of transient GUS expression in tobacco protoplasts. Each bar represents the averaged results of minimum of two measurements. The error bars represent standard deviations of the mean values. A. The experiment done on 04.04.01. B. The experiment done on 05.11.01.

B.

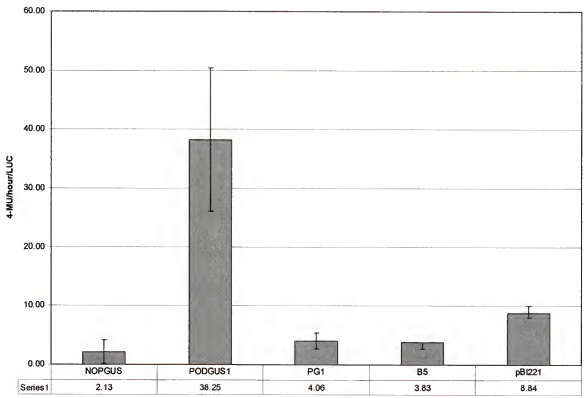


Figure 19 – continued.

introns, is the strongest of all POD3 promoter versions tested in tobacco protoplasts. The levels of gene expression regulation in eukaryotes may include transcription initiation, splicing, mRNA stability and translation. Only spliced POD3-GUS chimeric mRNA was detected in alfalfa carrying B5, a POD3-GUS construct with introns, suggesting efficient splicing. A study of Cherkaoui et al. (2000) on the haploid intergeneric hybrids suggest that splicing machinery of one plant species works in others. However, 5'- and 3'-end cryptic splicing sites can be a problem (Ibrahim et al., 2001). Ibrahim et al.(2001) removed the cryptic splice sites and the reporter gene expression was elevated. In this study, POD3-GUS constructs were not analyzed for aberrant splicing, but the POD3 gene has three GT dinucleotides at the positions 1287, 1291 and 1297 (see:Fig. 8), positioned less than 6 nt from the donor site of the first POD3 intron. There is a chance one of them can serve as an alternative donor splice site and disrupt the open reading frame of the gene. There is also one AG dinucleotide 4 nt downstream of the first intron's acceptor site, which may be an alternative splice acceptor site. There are no consensus GT and AG dinucleotides within 6 nt of distance from 5'- and 3'-end of the second POD3 intron, respectively. So, the aberrant splicing can be one of the explanations why POD3-GUS constructs containing the first POD3 intron are not as active as the intronless construct PODGUS1. The splicing may be incorrect due to different plant species, different tissue or due to using naked plasmid DNA introduced in protoplasts in high copy number in comparison to a gene on a chromosome. Messenger RNA stability seems unlikely to make these constructs to express GUS differently in protoplasts, because the mRNA stability is usually affected by sequences at the mRNA termini, and all POD3 constructs

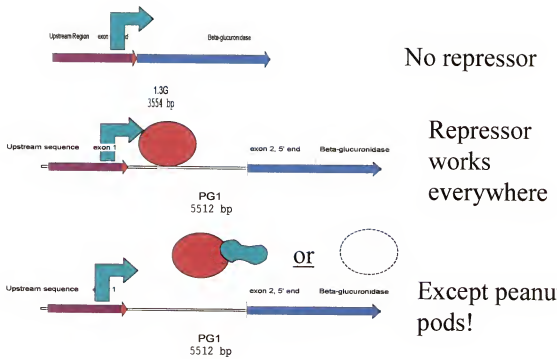


Figure 20. The silencer hypothesis.

have the same ends. The difference in translation rates is also unlikely because the translations of all constructs start from the same translation initiation site of POD3 (see Fig. 1). The constructs may be different in their rates of transcription if a negative transcriptional response element (silencer) is present in the first intron of POD3. The examples of tissue-specific silencers in the introns are numerous (Germain et al., 1999; Stark et al., 2001; Lum and Lee, 2001; Yan et al., 2001). A silencer in the first intron may be active in seeds but not pods, explaining the differential expression data on POD3 (see: Fig. 4). According to this model, pod-specific expression of POD3 in peanuts is to be achieved by differences in occupancy of the intron by a transcriptional repressor whose abundance differs between pods and seeds. In peanut seeds, it is occupied by a trans-acting protein, interacting with polymerase II and switching off transcription initiation or promoting pre-mature termination. In pods, it is absent from its binding site either because it is not present, or because it interacts with other trans-regulatoty factors preventing it from binding to the intron (Fig. 20). If the POD3 gene product is needed for response to changing environment, then switching its expression on by removing a repressor would be faster than activation by an enhancer. Silencers are thus thought to have an advantage over enhancers when fast response is needed.

CHAPTER 4
CONCLUSIONS

A strong, constitutive peanut promoter was cloned and characterized that should be very useful in genetic engineering of peanuts and other plants. That promoter was identified by differential display by comparing the expression of peanut pod and seed mRNAs. A differential display product, POD3, was selected that displayed strong expression in pod and no expression in seed mRNAs. Northern analyses revealed that POD3 was expressed very strongly in pod with some expression in seed coat, but no expression in seed or leaf tissues. The genomic counterpart of POD3 was screened from a partial Lambda ZAP II library of *XbaI*-digested genomic DNA fragments (about 7 kb). The selected 7 kb clone (named F5) contained POD3 sequence and 3.9 kb upstream of POD3 which contained promoter elements. Further research identified two transcription start sites for the POD3 gene that consisted of three exons (total 499 bp) and two large introns of 1938 bp and 635 bp.

Characterization of the promoter elements was hampered by the fact that peanuts are very difficult to transform and, to date, a repeatable transformation protocol does not exist. Two systems, transgenic alfalfa (also a legume) and tobacco protoplasts, were selected to characterize the promoter. Three promoter-GUS reporter constructs were assembled, PODGUS1, PG1 and B5, which included no introns, the first intron and two introns of POD3, respectively. B5 was stably introduced in alfalfa and GUS expression was detected. All constructs were transiently expressed in tobacco protoplasts and

PODGUS1 gave the GUS enzyme activity higher than that of 35S-GUS positive control construct. The construct with intron(s), PG1 and B5, were approximately 6-fold less active. This data indicates that POD3 promoter without introns is a very strong promoter and that the inclusion of the first or both introns down-regulates the promoter expression. To explain this, a model is presented in Fig. 20. Briefly, a silencer is hypothesized to be present in the POD3 first intron, repressing transcription in all tissues except pods. A repressor, a trans-acting protein, binding to the first intron in peanut seeds, is thought to interact with polymerase II and switching off transcription initiation or promoting pre-mature termination. In pods, it is absent from its binding site either due to some pod-specific regulatory mechanism which could be inhibition of its synthesis or interaction with another protein.

Experimental evidence of tissue specific gene expression by this promoter has not been established but the model is a reasonable hypothesis explaining POD3 tissue specific transcriptional expression in peanut. Should this promoter, when engineered into peanut, express that tissue specificity it will be of special value for engineering gene expression in pods to modify pod traits, but not be expressed in seeds. This could permit antifungal gene expression to prevent aflatoxin contamination of peanut, which causes serious human health problems worldwide. Absense of the antifungal gene product in seeds could alleviate health concerns of transgenic products seeds of a major worldwide food crop.

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BIOGRAPHICAL SKETCH

Denis Vadimovich Beliaev was born on April 22, 1970 in the town of Kaliningrad near Moscow, Russia. He entered the combined Bachelor of Science/Master of Science program of the Moscow Institute of Physics and Technology, Dolgoprudny, Russia, in September of 1987 and received his Master of Science degree in molecular biophysics in June of 1993 from this institution. In August of 1993, he entered the Ph. D. program in molecular genetics and microbiology at the University of Florida, and in August of 1994 transferred to the Ph. D. program in plant molecular and cellular biology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rex L. Smith
Professor of Plant Molecular and Cellular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Christine D. Chase
Associate Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John M. Davis
Associate Professor of Forest Resources and Conservation

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William B. Gurley
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William W. Hauswirth
Eminent Scholar of Molecular Genetics and Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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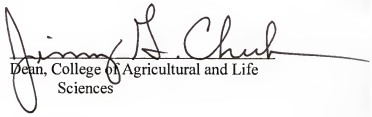

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